# Kongeriget Danmark

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**Patent- og Varemærkestyrelsen**
Økonomi- og Erhvervsministeriet

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Ella Vang M. Hansen

**PATENT- OG VAREMÆRKESTYRELSEN**

# Linear and hairpin oligonucleotide analogues comprising intercalator pseudonucleotides

## Field of the invention

The present invention relates to the field of pseudonucleotides comprising intercalators. In one embodiment the invention relates to the field of linear and hairpin oligonucleotide analogues comprising said intercalator pseudonucleotides In particular the invention relates to such oligonucleotide analogues for use in extension and amplification of nucleic acids and nucleic acid analogues.

Furthermore, the invention relates to the field of detecting hybridisation of said oligonucleotide analogues as well as to incorporation of said oligonucleotide analogues into nucleic acids or nucleic acid analogues and to detection of said intercalator pseudonucleotides in extension products.

## Background of invention

Nucleic acids, such as DNA, RNA as well as a number of nucleic acid analogues such as LNA, PNA, HNA, ANA, MNA, 2'-O-methyl RNA and others are capable of specifically hybridizing to their complementary strands. This specific recognition has for example been used to detect the presence of specific nucleic acid sequences and to prime extension of specific oligonucleotides or oligonucleotide analogues in a template dependent manner.

Certain synthetic nucleic acids have an increased affinity for nucleic acids in general. High affinity towards target nucleic acids may greatly facilitate hybridization based_assays and high affinity may also be useful for a number of other reactions involving nucleic acids for example amplification reactions.

Furthermore, most nucleic acids as well as most synthetic nucleic acid analogues do not discriminate rigidly between different kinds of nucleic acid, i.e. they bind roughly equally well to complementary DNA and complementary RNA.

Pyrene is an excimer-forming molecule, which has been incorporated into oligodeoxynucleotides (ODNs) by several groups. Ebata et al. incorporated a pyrene-modified nucleotide in the 5' end of one ODN and a pyrene-modified nucleotide into the 3' end of another. By hybridizing to a target sequence in a way that the pyrene moieties from the two strands come into close proximity of each other, an excimer band at 490 nm was generated. Paris et al. published a similar system were they also explored the utility of the system to detect mismatches, but the ability of the system to differentiate between a fully complementary sequence (wt) and a single point mutant (mut) is due to the ability of one of the probes to hybridize in the first case but not in the latter. This means that the phenomena is temperature controlled and limits the length of the probe and hence the selectivity and sets high requirements to the temperature control.

In the prior art synthetic nucleotide like molecules comprising intercalators have been described:

**US 5,446,578** describes synthetic nucleotide like molecules comprising fluorescent molecules, which show a change in spectra with concentration, for example pyrene. In particular, the document describes nucleic acids derivatised with such fluorescent molecule on the phosphate of a nucleic acid backbone or nucleic acids comprising an acyclic backbone monomer unit consisting of 5 atoms between two phosphates of the nucleic acid backbone, coupled to such a fluorescent molecule. The document states that the fluorescent molecules should be positioned at the exterior of a nucleic acid helix so that they are not capable of intercalating with nucleobases of a nucleic acid. Furthermore, it is explained that the fluorescence of the fluorescent molecule increases upon hybridization and that a cationic surfactant must be present to achieve this effect.

**Yamana et al., 1999,** describes an oligonucleotide containing a 2´-O-(1-pyrenylmethyl)uridine at the center position. Said oligonucleotide has higher affinity for DNA and lower affinity for RNA compared to an unmodified oligonucleotide. Upon hybridization monomer and exciplex fluorescence is enhanced.

**Yamana et al., 1997**, describes a phosphoramidit coupled to pyrene, which may be incorporated into a nucleic acid at any desired position. In particular said phosphoramidit may be incorporated into a nucleic acid, as an acyclic backbone monomer consisting of 5 atoms between two phosphates of the nucleic acid backbone. Upon hybridization, excimer fluorescence is greatly enhanced and nucleic acids into which said phosphoramidites have been incorporated retain normal binding affinity for DNA.

**Korshun et al., 1999**, describes a phosphoramidit coupled to a pyrene, which may be incorporated into a nucleic acid at any desired position. In particular said phosphoramidit may be incorporated into a nucleic acid, as an acyclic backbone monomer consisting of 5 atoms between two phosphates of the nucleic acid backbone. Furthermore the document describes oligonucleotides into which said phosphoramidits have been incorporated and it is described that the oligonucleotides have higher affinity for DNA, than an unmodified oligonucleotide. It is mentioned that close coplanar mutual approach of two pyrene residues located in the neighboring positions of a modified oligonucleotide chain is strongly inhibited because of the small length of the linker. Excimer fluorescence increases upon hybridization, however oligonucleotides comprising 5 such pyrene pseudonucleotides at the end exhibit high excimer fluorescence when unhybridised as well.

**US 5,414,077** describes pseudonucleotides, which may comprise an intercalator such as an acridine or anthraquinone. The pseudonucleotide comprises an achiral or a single enantiomer organic backbone, such as diethanolamine. The pseudonucleotides may be incorporated at any desired position within an oligonucleotide. Such oligonucleotides in general have higher affinity for complemenntary nucleotides, in particular when the pseudonucleotides are inserted at the end. The document does not describe fluorescence data.

**US 6,031,091** describes pseudonucleotides which may be incorporated at any position in an oligonucleotide. In particular the document describes acyclic phosphor containing backbones and it is mentioned that the pseudonucleotides may comprise an intercalator. Specific pseudonucleotides described in the document comprise very long linkers connecting polyaromates to the nucleic acid backbone.

**EP 0 916 737 A2** describes polynucleotides derivatised with for example intercalating compounds. The intercalating compounds should preferably be positioned with approx. 10 nucleotides in between. The polynucleotide may be derivatised on the phosphate, the sugar or the nucleobase moiety. In particular, they may be derivatised on the nucleobase by a 7 or a 11 atoms long linker coupled to a polyaromate in a manner that does not interfere with Watson-Crick base pairing. It is stated that fluorescence intensity is enhanced by intercalation.

**Strässler et al., 1999**, describes pseudonucleosides comprising a fluorescent molecule for example pyrene instead of a nucleobase.

**WO 97/43298** describes nucleoside analogues comprising a polyaromatic hydrocarbon for example pyrene attached to the 1' position of ribose or deoxyribose as well as phosphoramidite derivatives of said polyaromatic hydrocarbons.

**US 5,175,273** describes nucleotide derivatives comprising nucleobases fused to planar polycyclic aromatic compounds. Oligonucleotide comprising said nucleotide derivatives have increased affinity for DNA and fluorescence is decreased by hybridization.

**US 6,153,737** describes nucleotide derivatised on the 2' position with pyrene or another intercalator.

**Ebata et al., 1995** describes incorporation of a pyrene-modified nucleotide in the 5' end of a DNA oligonucleotide and a pyrene-modified nucleotide into the 3' end of another. By hybridizing to a target sequence in a way that the pyrene moieties from the two strands come into close proximity of each other, an excimer band at 490 nm was generated.

**Paris et al., 1998** described a system similar to the one disclosed by Ebata et al. wherein the system may be utilized to detect mismatches. However the ability of the system to differentiate between a fully complementary sequence (wt) and a single point mutant (mut) is due to the ability of one of the probes to hybridize in the first case but not in the latter. This means that the phenomena is temperature controlled and limits the length of the probe and hence the selectivity and sets high requirements to the temperature control.

In the prior art nucleotides and nucleotides analogues capable of forming hairpin structures have been described:

Hairpin-forming oligonucleotide hybridization probes with interactive label pairs, particularly fluorescent label pairs and fluorescence-quencher label pairs, are known. Tyagi et al., PCT application No. WO95/13399; Tyagi et al., PCT application No. WO97/39008; Tyagi and Kramer (1996) Nature Biotechnology 14:303. These probes, labeled with a fluorophore and a quencher, are usually "dark", that is, have relatively little or no fluorescence, when free in solution but fluoresce when hybridized to their nucleic acid targets. They are constructed with a variety of fluorophores and are utilized in both end-point and real-time homogeneous assays, including multiplex assays. Tyagi et al. (1998) Nature Biotechnology 16:49; Kostrikis et al. (1998) Science 279:1228; Piatek et al. (1998) Nature Biotechnology 16:359. Hairpin-containing primers similarly labeled with a fluorophore and a quencher are also known. Nazarenko et al. (1997) Nucleic Acids Research 25:2516.

## Summary of the invention

End point detection and real time detection of oligonucleotide or oligonucleotide analogue template directed extension is useful for a large number of different applications. However, there has been a shortage of easy-to-handle, readily available, specific, and cost-effective detection methods with fast kinetics that permit real quantitative and real-time detection.

Surprisingly, the inventors have discovered, that the insertion of intercalators into oligonucleotides or oligonucleotide analogues results in spectral and thermodynamic properties, which allow for specific hybridization, which may be followed by detection of template directed extension of oligonucleotide analogues.

Accordingly, it is a first object of the present invention to provide hairpin oligonucleotide analogues comprising at least one intercalator pseudonucleotide of the general structure

X-Y-Q

wherein

X is a backbone monomer unit capable of being incorporated into the phosphate backbone of a nucleic acid; and

Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid; and

Y is a linker moiety linking said backbone monomer unit and said intercalator; and

and wherein the oligonucleotide comprises a first sequence consisting of n nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides and a second nucleotide sequence consisting of m nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotide, wherein said first sequence is capable of hybridising to said second sequence.

In particular, it is an object of the present invention to provide such hairpin oligonucleotide analogues, wherein said first sequence and said second sequence are separated by a third sequence consisting of p nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides, wherein said first sequence is capable of hybridizing to said second sequence; and wherein the second and third sequence is capable of hybridizing to a corresponding target sequence comprised within a nucleic acid or nucleic acid analogue and is capable of priming a template directed extension reaction.

It is also an object of the present invention to provide methods for detecting template directed extension of an oligonucleotide analogue comprising intercalator pseudonucleotides

a) providing the template(s); and
b) providing at least one oligonucleotide analogue comprising intercalator pseudonucleotide(s); and
c) incubating said template(s) and said oligonucleotide analogue under conditions allowing for hybridization; and
d) providing nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides; and
e) extending the oligonucleotide analogue in a templated directed manner; and
f) detecting said template directed extension.

Furthermore, it is an object of the present invention to provide methods of detecting hybridisation between an hairpin oligonucleotide analogue comprising an intercalator pseudonucleotide as described herein above and a template comprising a nucleic acid sequence capable of hybridising to the second sequence of said hairpin oliognucleotide analogue comprising the steps of

a) providing the template; and
b) providing an oligonucleotide or oligonucleotide analogue according to the present invention; and
c) incubating the template and the oligonucleotide or oligonucleotide analogue under conditions allowing for hybridisation; and
d) detecting hybridisation.

## Legends to figures

**Figure 1** illustrates the synthesis of an intercalator pseudonucleotide, a phosphoramidite as depited in 5.

**Figure 2** illustrates a structural calculation of the self-complementary DNA duplex with the sequence 5'-XCGCGCG-3' done in "MacroModel", X = the pyrene module. The pyrene moiety is co-axial stacked with the underlying base pair.

**Figure 3** illustrates fluorescent measurements of a 13-mer, mono pyrene inserted ssDNA (★); its duplex with complementary, 12-mer RNA ( ) and its duplex with complementary, 12-mer DNA (◆). The sequences are the same as those shown in Table 3.

**Figure 4** illustrates fluorescent measurements of a 14-mer ssDNA with two pyrene insertions separated by one nucleotide (★); its duplex with complementary, 12-mer RNA ( ) and its duplex with complementary, 12-mer DNA (◆).The sequences are the same as those shown in Table 3.

**Figure 5** illustrates a Ubeacon primer

**Figure 6** illustrates a PCR quantification strategy using Ubeacon primers

## Detailed description of the invention

Nucleotides, Oligonucleotides and Nucleic acids

The term nucleotide designates the building blocks of oligonucleotides or oligonucleotide analogues or nucleic acids or nucleic acid analogues and covers all naturally occurring nucleotides. The term "nucleotide analogue" covers any nucleotide like molecule that is capable of being incorporated into a nucleic acid backbone and capable of specific base-pairing, for example a nucleotide analogue may be a derivative of natural nucleotides which is capable of performing some of the same functions, as naturally occurring nucleotides for example like base-pairing. Naturally occurring nucleotides comprise deoxyribonucleotides comprising one of the four nucleobases adenine (A), thymine (T), guanine (G) or cytosine (C), and ribonucleotides comprising on of the four nucleobases adenine (A), uracil (U), guanine (G) or cytosine (C).

Nucleotide analogues may be any nucleotide like molecule that is capable of being incorporated into a nucleic acid backbone and capable of specific base-pairing. Hence nucleotide analogues according to the present invention includes, but is not limited to the subunits comprised within LNA, 2'-O-METHYL RNA, PNA, MNA, ANA and HNA.

Pseudonucleotides may be any nucleotide like molecule that is capable of being incorporated into a nucleic acid backbone but cannot make specific base-pairing. Pseudonucleotides are preferably incorporated chemically into the growing chain of an oligonucleotide or oligonucleotide analogue.

Nucleotides or nucleotide analogues comprise a backbone monomer unit and a nucleobase. The nucleobase may be a naturally occurring nucleobase or a derivative thereof or an analogue thereof capable of performing essentially the same function. The function of a nucleobase is to be capable of associating specifically with one or more other nucleobases via hydrogen bonds. Thus it is an important feature of a nucleobase that it can only form stable hydrogen bonds with one or a few other nucleobases, but that it can not form stable hydrogen bonds with most other nucleobases usually including itself. The specific interaction of one nucleobase with another nucleobase is generally termed "base-pairing".

The function of nucleotides and nucleotide analogues according to the present invention is to be able to interact specifically with complementary nucleotides via hydrogen bonding of the nucleobases of said complementary nucleotides as well as to be able to be incorporated into a nucleic acid or nucleic acid analogue. Naturally occurring nucleotides, as well as some nucleotide analogues are capable of being enzymatically incorporated into a nucleic acid or nucleic acid analogue, for example by RNA or DNA polymerases, however nucleotides or nucleotide analogues may also be chemically incorporated into a nucleic acid or nucleic acid analogue.

Base-pairing results in a specific hybridization between complementary nucleotides or nucleotide analogues. Complementary nucleotides according to the present invention are nucleotides that comprise nucleobases that are capable of base-pairing.

Of the naturally occurring nucleobases adenine (A) pairs with thymine (T) or uracil (U); and guanine (G) pairs with cytosine (C). Accordingly, e.g. a nucleotide comprising A is complementary to a nucleotide comprising either T or U, and a nucleotide comprising G is complementary to a nucleotide comprising C.

Nucleotides according to the present invention may further be derivatised to comprise an appended molecular entity. The nucleotides can be derivatised on the nucleobases or on the backbone monomer unit. Preferred sites of derivatisation on the bases include the 8-position of adenine, the 5-position of uracil, the 5- or 6-position of cytosine, and the 7-position of guanine. The heterocyclic modifications may be grouped into three structural classes: Enhanced base stacking, additional hydrogen bonding and the combination of these. Modifications that enhance base stacking by expanding the π-electron cloud of the planar systems are represented by conjugated, lipophilic modifications in the 5-position of pyrimidines and the 7-position of 7-deaza-purines. Substitutions in the 5-position of pyrimidines modifications include propynes, hexynes, thiazoles and simply a methyl group; and substituents in the 7-position af 7-deaza purines include iodo, propynyl, and cyano groups. It is also possible to modify the 5-position of cytosine from propynes to five-membered heterocycles and to tricyclic fused systems, which emanate from the 4- and 5-position (cytosine clamps). A second type of heterocycle modification is represented by the 2-amino-adenine where the additional amino group provides another hydrogen bond in the A-T base pair, analogous to the three hydrogen bonds in a G-C base pair. Heterocycle modifications providing a combination of effects are represented by 2-amino-7-deaza-7-modified andenine and the tricyclic cytosine analog having an ethoxyamino functional group of heteroduplexes. Furthermore, N2-modified 2-amino adenine modified oligonucleotides are among commonly modifications. Preferred sites of derivatisation on ribose or deoxyribose moieties are modifications of nonconnecting carbon positions C-2' and C-4', modifications of connecting carbons C-1', C-3' and C-5', replacement of sugar oxygen, O-4', Anhydro sugar modifications (conformational restricted), cyclosugar modifications (conformational restricted), ribofuranosyl ring size change, connection sites – sugar to sugar, (C-3' to C-5'/ C-2' to C-5'), hetero-atom ring – modified sugars and combinations of above modifications._However, other sites may be derivatised, as long as the overall base pairing specificity of a nucleic acid or nucleic acid analogue is not disrupted.

Finally, when the backbone monomer unit comprises a phosphate group, the phosphates of some backbone monomer units may be derivatised. Such modifications of the phosphorous atom includes, but are not limited to phosphorothioates, methyl phospholates, phosphoramidiates, phosphorodithiates, phosphoroselenoates, phosphotriesters and phosphoboranoates. In addition non-phosphorous containing compounds can be used for linking to nucleotides such as but not limited to methyliminomethyl, formacetate, thioformacetate and linking groups comprising amides.

Pseudonucleotides may be any nucleotide like molecule that is capable of being incorporated into a nucleic acid backbone. Pseudonucleotides are preferably incorporated chemically into the growing chain of an oligonucleotide or oligonucleotide analogue.

Oligonucleotides or oligonucleotide analogues as used herein are molecules essentially consisting of a sequence of nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides. Preferably oligonucleotides or oligonucleotide analogues comprise 5 to 100 individual nucleotides. Oligonucleotides or oligonucleotide analogues may comprise DNA, RNA, LNA, 2'-O-METHYL RNA, PNA, MNA, ANA, HNA and mixtures thereof, as well as any other nucleotides and/or nucleotide analogues and/or pseudonucleotides. According to the present invention oligonucleotide analogues may comprise one or more intercalator pseudonucleotide(s).

The term “nucleic acid” covers the naturally occurring nucleic acids, DNA and RNA. The term “nucleic acid analogue” covers the synthetic analogues of naturally occurring nucleic acids. Synthetic analogues comprise one or more nucleotide analogues. Hence nucleic acids and nucleic acid analogues also includes oligonucleotides and oligonucleotide analogues.

Nucleic acids and nucleic acid analogues may be synthesised by different methods for example they may be synthesised enzymatically by polymerases or chemically. Furthermore nucleic acids or nucleic acid analogues may be prepared by coupling two smaller nucleic acids or nucleic acid analogues to another, for example this may be done enzymatically by ligases or it may be done chemically.

Hence the terms “nucleic acid” or “nucleic acid analogue” designates any molecule, which essentially consists of a plurality of nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides. Intercalator pseudonucleotides are described in detail herein below. Nucleic acids or nucleic acid analogues according to the present invention may comprise mixtures of different nucleotides and/ or nucleic acid analogues with different backbone monomer units.

Preferably, single strands of nucleic acids or nucleic acid analogues according to the present invention are capable of hybridizing with a substantially complementary single stranded nucleic acid and/or nucleic acid analogue to form a double stranded nucleic acid or nucleic acid analogue. More preferably such a double stranded analogue is capable of forming a double helix. Preferably, the double helix is formed due to hydrogen bonding; more preferably, the double helix is a double helix of A-, B- or Z-form or intermediates thereof.

Hence, nucleic acids and nucleic acid analogues according to the present invention includes, but is not limited to DNA, RNA, LNA, 2'-*O*-methyl RNA, PNA, MNA, ANA, HNA and mixtures thereof and hybrids thereof and their phosphorous atom modifications such as but not limited to phosphorothioates, methyl phospholates, phosphoramidiates, phosphorodithiates, phosphoroselenoates, phosphotriesters and phosphoboranoates. In addition non-phosphorous containing compounds can be used for linking to nucleotides such as but not limited to methyliminomethyl, formacetate, thioformacetate and linking groups comprising amides.

Within this context, mixture is meant to cover a nucleic acid or nucleic acid analogue strand comprising different kinds of nucleotides or nucleotide analogues. Furthermore, within this context, hybrid is meant to cover a double helix comprised of two strands of different types of nucleotides or nucleotide analogues or where at least one strand is comprised of a mixture of nucleotides or nucleotide analogues.

By HNA is meant nucleic acids as for example described by Van Aetschot et al., 1995. By MNA is meant nucleic acids as described by Hossain et al, 1998. ANA refers to nucleic acids described by Allert et al, 1999. LNA may be any LNA molecule as described in WO 99/14226 (Exiqon), preferably, LNA is selected from the molecules depicted in the abstract of WO 99/14226. More preferably LNA is a nucleic acid as described in Singh et al, 1998, Koshkin et al, 1998 or Obika et al.,

1997. PNA refers to peptide nucleic acids as for example described by Nielsen et al., 1991. 2'-O-methyl RNA is described by Inoue et al. 1987.

Preferred oligonucleotides or oligonucleotide analogues according to the present invention will be described in more detail herein below.

Nucleic acids and nucleic acid analogues, including oligonucleotides and oligonucleotide analogues, are said to be orientated from the 5' to the 3' end. The 3' end of a nucleic acid or nucleic acid analogue comprises the site capable of being extended. The 5' end or a region close to the 5' end of a nucleic acid or a nucleic acid analogue is also referred to as "upstream" whereas the 3' end or a region close to the 3' end is referred to as "downstream".

The term "template" according to the present invention refers to a nucleic acid or nucleic acid analogue. Preferably a template comprises a nucleotide sequence that corresponds to a specific oligonucleotide or oligonucleotide analogue. If said sequence comprised within said template is internally positioned, hybridization between said template and said oligonucleotide or oligonucleotide analogue will create a 3' free end of said oligonucleotide or oligonucleotide analogue. This 3' free end is capable of being extended by a for example a DNA polymerase in a template directed manner and said oligonucleotide or oligonucleotide analogue is thus said to prime extension. The term "primer" therefore covers a oligonucleotide or oligonucleotide analogue that is able to prime template directed extension by DNA and RNA polymerases.

Cross-hybridisation

The term cross-hybridisation covers unattended hybridisation between at least two nucleic acids or nucleic acid analogues. Hence the term cross-hybridization may be used to describe the hybridisation of for example a nucleic acid probe or nucleic acid analogue probe sequence to other nucleic acid sequences or nucleic acid analogue sequences than its intended target sequence.

Often cross-hybridization occurs between a probe and one or more corresponding non-target sequences, even though these have a lower degree of complementarity than the probe and its corresponding target sequence. This unwanted effect could be due to a large excess of probe over target and/or fast annealing kinetics. Cross-hybridization also occurs by hydrogen bonding between few nucleobase pairs, e.g. between primers in a PCR reaction, resulting in primer dimer formation and/ or formation of unspecific PCR products.

Especially nucleic acids comprising one or more nucleotide analogues with high affinity for nucleotide analogues of the same type tend to form dimer or higher order complexes based on base pairing. Especially probes comprising nucleotide analogues such as, but not limited to, LNA, 2'-O-METHYL RNA and PNA generally have a high affinity for hybridising to other oligonucleotide analogues comprising backbone monomer units of the same type. Hence even though individual probe molecules only have a low degree of complementarity they tend to hybridise.

## Self-hybridisation

The term self-hybridisation covers the process wherein a nucleic acid or nucleic acid analogue molecule anneals to itself by folding back on itself, generating a secondary structure like for example a hairpin structure.

Accordingly, the term hairpin oligonucleotide or oligonucleotide analogue covers any oligonucleotide or oligonucleotide analogue capable of self-hybridisation, i.e. any oligonucleotide or oligonucleotide analogue comprising two individual sequence parts, which may hybridise to one another.

## Corresponding nucleic acids

Nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotides analogues are said to be corresponding, when they are capable of hybridising. Preferably corresponding nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotides analogues are capable of hybridising under low stringency conditions, more preferably corresponding nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotides analogues are capable of hybridising under medium stringency conditions, even more preferably corresponding nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotides analogues are capable of hybridising under high stringency conditions.

High stringency conditions as used herein shall denote stringency as normally applied in connection with Southern blotting and hybridisation as described e.g. by Southern E. M., 1975, J. Mol. Biol. 98:503-517. For such purposes it is routine practise to include steps of prehybridization and hybridization. Such steps are normally performed using solutions containing 6x SSPE, 5% Denhardt's, 0.5% SDS, 50% formamide, 100 $\mu$g/ml denaturated salmon testis DNA (incubation for 18 hrs at 42°C), followed by washings with 2x SSC and 0.5% SDS (at room temperature and at 37°C), and washing with 0.1x SSC and 0.5% SDS (incubation at 68°C for 30 min), as described by Sambrook et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor), which is incorporated herein by reference.

Medium stringency conditions as used herein shall denote hybridisation in a buffer containing 1 mM EDTA, 10mM Na2HPO4.H20, 140 mM NaCl, pH 7.0. Preferably, around 1.5 $\mu$M of each nucleic acid or nucleic acid analogue strand is provided.

Alternatively medium stringency shall denote hybridisation in a buffer containing 50 mM KCl, 10 mM TRIS-HCl (pH 9,0), 0.1% Triton X-100, 2 mM $MgCl_2$.

Low stringency conditions according to the present invention denote hybridisation in a buffer constituting 1 M NaCl, 10 mM $Na_3PO_4$ at pH 7,0 .

Alternatively, corresponding nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotides are nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotides substantially complementary to each other over a given sequence, such as more than 70% complementary, for example more than 75% complementary, such as more than 80% complementary, for example more than 85% complementary, such as more than 90% complementary, for example more than 92% complementary, such as more than 94% complementary, for example more than 95% complementary, such as more than 96% complementary, for example more than 97% complementary.

Preferably said given sequence is at least 10 nucleotides long, such as at least 15 nucleotides, for example at least 20 nucleotides, such as at least 25 nucleotides, for example at least 30 nucleotides, such as between 10 and 500 nucleotides, for example between 10 and 100 nucleotides long, such as between 10 and 50 nucleotides long. More preferably corresponding oligonucleotides or oligonucleotides analogues are substantially complementary over their entire length.

## Melting temperature

Melting of nucleic acids refer to the separation of the two strands of a double-stranded nucleic acid molecule.
The melting temperature ($T_m$) denotes the temperature in degrees centigrade at which 50% helical (hybridized) versus coil (unhybridized) forms are present.

A high melting temperature is indicative of a stable complex and accordingly of a high affinity between the individual strands. Vice versa a low melting temperature is indicative of a relatively low affinity between the individual strands. Accordingly, usually strong hydrogen bonding between the two strands results in a high melting temperature.

Furthermore, as disclosed by the present invention, intercalation of an intercalator between nucleobases of a double stranded nucleic acid may also stabilise double stranded nucleic acids and accordingly result in a higher melting temperature.

In addition the melting temperature is dependent on the physical/chemical state of the surroundings. For example the melting temperature is dependent on salt concentration and pH.

The melting temperature may be determined by a number of assays, for example it may be determined by using the UV spectrum to determine the formation and breakdown (melting) of hybridization.

## Backbone monomer unit

The backbone monomer unit of a nucleotide or a nucleotide analogue or an intercalator pseudonucleotide according to the present invention is the part of the nucleotide, which is involved in incorporation of the nucleotide or nucleotide analogue or intercalator pseudonucleotide into the backbone of a nucleic acid or a nucleic acid analogue. Any suitable backbone monomer unit may be employed with the present invention.

In particular the backbone monomer unit of intercalator pseudonucleotides according to the present invention may be selected from the backbone monomer units mentioned herein below.

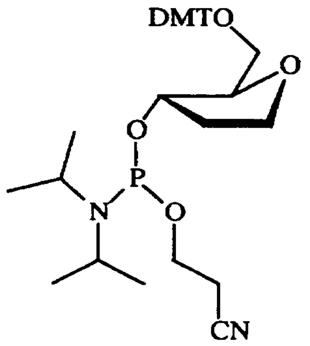
The backbone monomer unit comprises the part of a nucleotide or nucleotide analogue or intercalator pseudonucleotide which may be incorporated into the backbone of a nucleic acid or a nucleic acid analogue. In addition, the backbone monomer unit may comprise one or more leaving groups, protecting groups and/or reactive groups, which may be removed during synthesis or which alternatively may be removed subsequent to synthesis.

It is important to note that the term backbone monomer unit according to the present invention only includes the backbone monomer unit per se and it does not include for example a linker connecting a backbone monomer unit to an intercalator. Hence, the intercalator as well as the linker is not part of the backbone monomer unit. Accordingly, backbone monomer units only include atoms, which are selected from the group consisting of

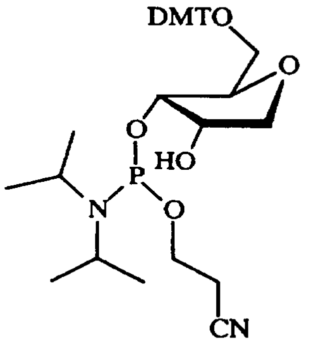
a) atoms which are capable of forming a linkage to the backbone monomer unit of a neighboring nucleotide; or
b) atoms which at least at two sites are connected to other atoms of the backbone monomer unit; or
c) atoms which at one site is connected to the backbone monomer unit and otherwise is not connected with other atoms

The backbone monomer unit may be any suitable backbone monomer unit. In one embodiment of the present invention, the backbone monomer unit may for example be selected from the group consisting of the backbone monomer units of DNA, RNA, LNA, 2'-O-METHYL RNA, PNA, MNA, HNA or ANA.

The backbone monomer unit of DNA consists of a deoxyribose and a phosphate group and has the following structure:



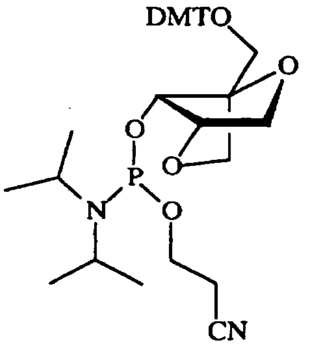
The backbone monomer unit consists of a ribose group and a phosphate group and has the following structure:



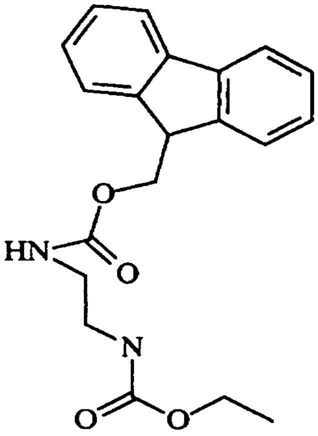
The backbone monomer unit of LNA (locked nucleic acid) is a sterically restricted DNA backbone monomer unit, which comprises an intramolecular bridge that restricts the usual conformational freedom of a DNA backbone monomer unit. LNA may be any LNA molecule as described in WO 99/14226 (Exiqon), preferably, LNA is selected from the molecules depicted in the abstract of WO 99/14226. Preferred LNA according to the present invention comprises a methyl linker connecting the 2'-

O position to the 4'-C position, however other LNA's such as LNA's wherein the 2' oxy atom is replaced by either nitrogen or sulphur are also comprised within the present invention.

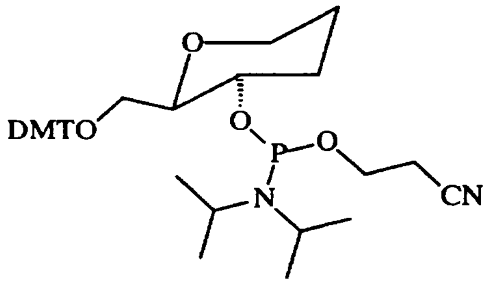
Preferably the backbone monomer unit of LNA has the following structure:



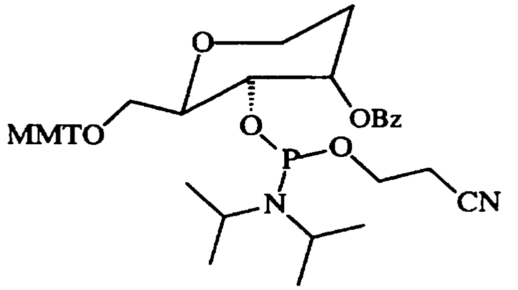
The backbone monomer unit of PNA (Peptide nucleic acid) is an amid backbone of the following structure:



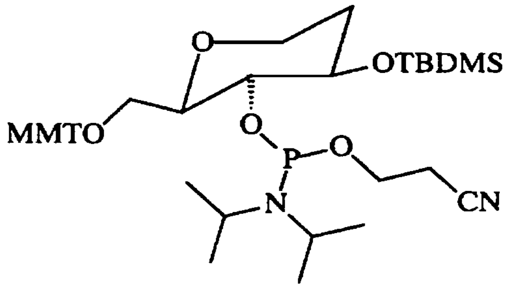
The backbone monomer unit of HNA has the following structure:



The backbone monomer unit of ANA has the following structure:



The backbone monomer unit of MNA has the following structure:



In one preferred embodiment of the present invention the backbone monomer unit of an intercalator pseudonucleotide is selected from the group consisting of acyclic backbone monomer units. Acyclic is meant to cover any backbone monomer unit, which does not comprise a ringstructure, for example the backbone monomer unit preferably does not comprise a ribose or a deoxyribose group.

In particular, it is preferred that the backbone monomer unit of an intercalator pseudonucleotide is an acyclic backbone monomer unit, which is capable of stabilising a bulge insertion (see herein below).

In another preferred embodiment the backbone monomer unit of an intercalator pseudonucleotide according to the present invention may be selected from the group consisting of backbone monomer units comprising at least one chemical group selected from the group consisting of phosphate, phosphoester, phosphodiester, phosphoramidate and phosphoramidit groups.

In particular it is preferred that the backbone monomer unit of a intercalator pseudonucleotide according to the present invention is selected from the group consisting of acyclic backbone monomer units comprising at least one chemical group selected from the group consisting of phosphate, phosphoester, phosphodiester, phosphoramidate and phosphoramidit groups.

Accordingly, the backbone monomer unit of an intercalator pseudonucleotide according to the present invention may comprise a phosphodiester. Additionally, the backbone monomer unit of an intercalator pseudonucleotide according to the present invention may comprise a pentavalent phosphoramidate. Preferably, the backbone monomer unit of an intercalator pseudonucleotide according to the present invention is an acyclic backbone monomer unit which may comprise a pentavalent phosphoramidate.

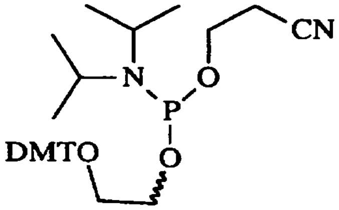
Preferred backbone monomer units comprising at least one chemical group selected from the group consisting of phosphate, phosphoester, phosphodiester, phosphoramidate and phosphoramidit groups are backbone monomer units, wherein the distance from at least one phosphor atom to at least one phosphor atom of a neighbouring nucleotide is at the most 6 atoms long, for example 2, such as 3, for example 4, such as 5, for example 6 atoms long, when the backbone monomer unit is incorporated into a nucleic acid backbone.

Hence, preferably the backbone monomer unit is capable of being incorporated into a phosphate backbone of a nucleic acid or nucleic acid analogue in a manner so that at the most 6 atoms are separating the phosphor atom of the intercalator pseudonucleotide backbone monomer unit and the nearest neighbouring phosphor atom, more preferably 4 atoms are separating the phosphor atom of the intercalator pseudonucleotide backbone monomer unit and the nearest neighbouring phosphor atom.

In a particularly preferred embodiment of the present invention the intercalator pseudonucleotide comprises a backbone monomer unit that comprises a phosphoramidit and more preferably the backbone monomer unit comprises a trivalent phosphoramidit.

Suitable trivalent phosphoramidits are trivalent phosphoramidits that may be incorporated into the backbone of a nucleic acid and/or a nucleic acid analogue. Usually, the amidit group per se may not be incorporated into the backbone of a nucleic acid, but rather the amidit group or part of the amidit group may serve as a leaving group. However, it is preferred that the backbone monomer unit comprises a phosphoramidit group, because such a group may facilitate the incorporation of the backbone monomer unit into a nucleic acid backbone.

Preferably, the acyclic backbone monomer unit may be selected from the group consisting of the structures a) to m) as indicated herein below:

a) 

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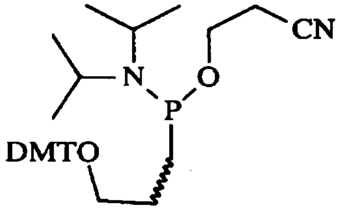
b) 

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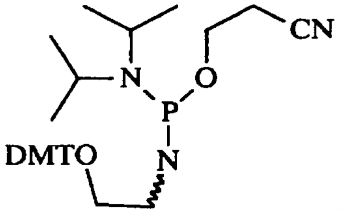
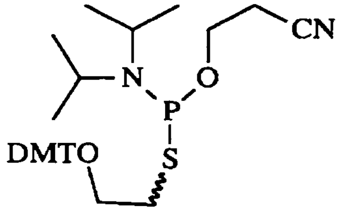
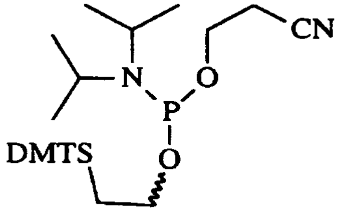
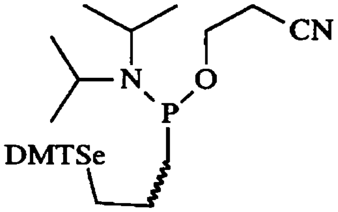
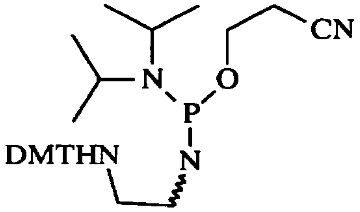
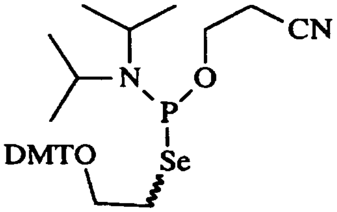
c)  ,

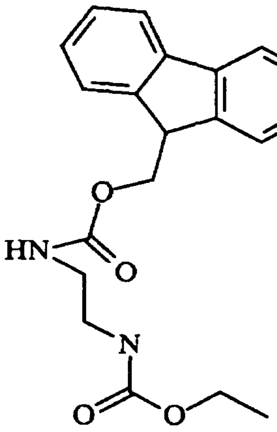
d)  ,

e)  ,

f)  ,

g)  ,

h)  , i)



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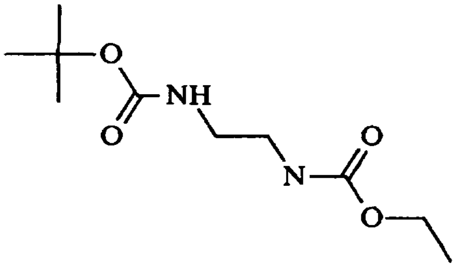
j)



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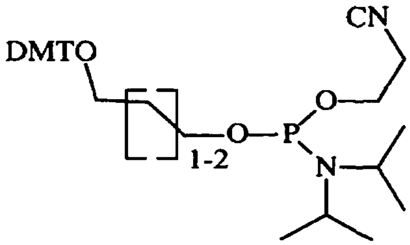

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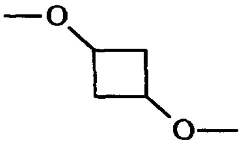

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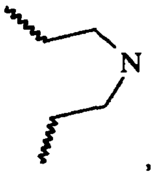
m)



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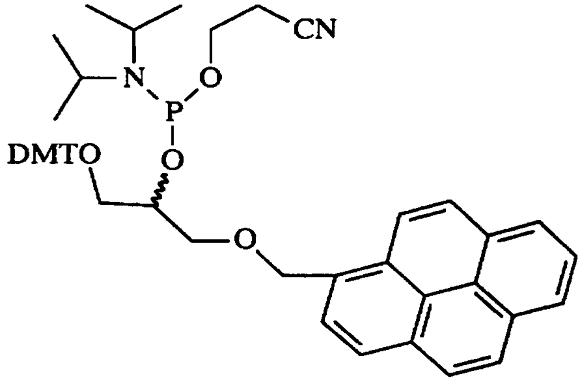
wherein DMT denotes dimethoxytrityl, which serves as a protecting group.

More preferably the trivalent phosphoramidit has the structure:



## Leaving group

The backbone monomer unit according to the present invention may comprise one or more leaving groups. Leaving groups are chemical groups, which are part of the backbone monomer unit when the intercalator pseudonucleotide or the nucleotide is a monomer, but which are no longer present in the molecule once the intercalator pseudonucleotide or the nucleotide has been incorporated into an oligonucleotide or oligonucleotide analogue.

The nature of a leaving group depends of the backbone monomer unit. For example, when the backbone monomer unit is a phosphor amidit, the leaving group may for example be a diisopropylamine group. In general, when the backbone monomer unit is a phosphor amidit, a leaving group is attached to the phosphor atom (e.g. in the form of diisopropylamine) and is lost from the product on coupling of the phosphor atom to a nucleophilic group, whereas the rest of the phosphate group, becomes part of the nucleic acid or nucleic acid analogue backbone.

## Reactive group

The backbone monomer units according to the present invention may furthermore comprise a reactive group, which is capable of performing a chemical reaction with another nucleotide or oligonucleotide or nucleic acid or nucleic acid analogue to form a nucleic acid or nucleic acid analogue, which is one nucleotide longer than before the reaction

Accordingly, when nucleotides are in their free form, i.e. not incorporated into a nucleic acid, the reactive group capable of reacting with another nucleotide or a nucleic acid or nucleic acid analogue may be protected by a protecting group. Prior to said chemical reaction, said protection group have to be removed. The protection group thus will not be a part of the newly formed nucleic acid or nucleic acid analogue.

Examples of reactive groups are nucleophiles such as the 5'-hydroxy group of DNA or RNA backbone monomer units.

Protecting group

The backbone monomer unit according to the present invention may also comprise a protecting group, which can be removed, and wherein removal of the protecting group allows for a chemical reaction between the intercalator pseudonucleotide and a nucleotide or nucleotide analogue or another intercalator pseudonucleotide.

In particular, a nucleotide monomer or nucleotide analogue monomer or intercalator pseudonucleotide monomer may comprise a protecting group, which is present in the molecule when the nucleotide or nucleotide analogue or intercalator pseudonucleotide is incorporated into a nucleic acid, but is no longer present after introduction of an additional nucleotide or nucleotide analogue or which is removed after the synthesis of the nucleotide or nucleotide analogue.

The protecting group may be removed by a number of suitable techniques known to the person skilled in the art, however preferably, the protecting group may be removed by a treatment selected from the group consisting of acid treatment, thiophenol treatment and alkali treatment.

Preferred protecting groups according to the present invention, which may be used to protect the 5' end or the 5' end analogue of a backbone monomer unit may be selected from the group consisting of trityl, monomethoxytrityl, 2-chlorotrityl, 1,1,1,2-

tetrachloro-2,2-bis(p-methoxyphenyl)-ethan (DATE), 9-phenylxanthine-9-yl (pixyl) and 9-(p-methoxyphenyl) xanthine-9-yl (MOX) or other protecting groups mentioned in "Current Protocols In Nucleic Acid Chemistry" volume 1, Beaucage et al. Wiley. More preferably the protecting group may be selected from the group consisting of monomethoxytrityl and dimethoxytrityl. Most preferably, the protecting is 4, 4'-dimethoxytrityl (DMT group).

4, 4'-dimethoxytrityl (DMT) groups may be removed by acid treatment, for example by brief incubation (30 to 60 seconds sufficient) in 3% trichloroacetic acid or in 3% dichlororacetic acid in $CH_2Cl_2$.

Preferred protecting groups which may protect a phosphate or phosphoramidit group of a backbone monomer unit may for example be selected from the group consisting of methyl and 2-cyanoethyl. Methyl protecting groups may for example be removed by treatment with thiophenol or disodium 2-carbamoyl 2-cyanoethylene-1,1-dithiolate. 2-cyanoethyl-groups may be removed by alkali treatment, for example treatment with concentrated aqueous ammonia.

## Intercalator

The term intercalator according to the present invention covers any molecular moiety comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid. Preferably an intercalator according to the present invention essentially consists of at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid.

Preferably, the intercalator comprises a chemical group selected from the group consisting of polyaromates and heteropolyaromates an even more preferably the intercalator essentially consists of a polyaromate or a heteropolyaromate. Most preferably the intercalator is selected from the group consisting of polyaromates and heteropolyaromates.

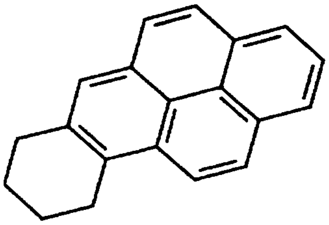
Polyaromates or heteropolyaromates according to the present invention may consist of any suitable number of rings, such as 1, for example 2, such as 3, for example 4, such as 5, for example 6, such as 7, for example 8, such as more than 8. Furthermore polyaromates or heteropolyaromates may be substituted with one or more selected from the group consisting of hydroxyl, bromo, fluoro, chloro, iodo, mercapto, thio, cyano, alkylthio, heterocycle, aryl, heteroaryl, carboxyl, carboalkoyl, alkyl, alkenyl, alkynyl, nitro, amino, alkoxyl and amido.

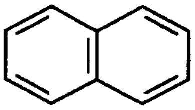
In one preferred embodiment of the present invention the intercalator may be selected from the group consisting of polyaromates and heteropolyaromates that are capable of forming excimers, exciplexes, fluorescence resonance energy transfer (FRET) or charge-transfer complexes.

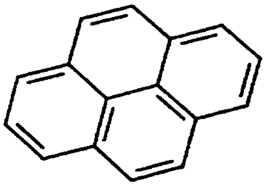
Accordingly, the intercalator may preferably be selected from the group consisting of phenanthroline, phenazine, phenanthridine, anthraquinone, pyrene, anthracene, napthene, phenanthrene, picene, chrysene, naphtacene, acridones, benzanthracenes, stilbenes, oxalo-pyridocarbazoles, azidobenzenes, porphyrins, psoralens and any of the aforementioned intercalators substituted with one or more selected from the group consisting of hydroxyl, bromo, fluoro, chloro, iodo, mercapto, thio, cyano, alkylthio, heterocycle, aryl, heteroaryl, carboxyl, carboalkoyl, alkyl, alkenyl, alkynyl, nitro, amino, alkoxyl and/or amido.

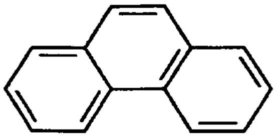
Preferably, the intercalator is selected from the group consisting of phenanthroline, phenazine, phenanthridine, anthraquinone, pyrene, anthracene, napthene, phenanthrene, picene, chrysene, naphtacene, acridones, acridines, benzanthracenes, stilbenes, oxalo-pyridocarbazoles, azidobenzenes, porphyrins and psoralens.

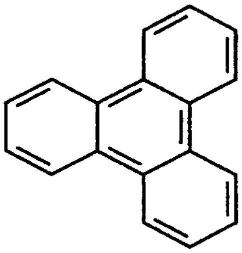
More preferably the intercalator may be selected from the group of intercalators comprising structures i) to xxx) as indicated herein below, more preferably the intercalators may be selected from the group consisting of the structure i) to xxx) as indicated herein below:

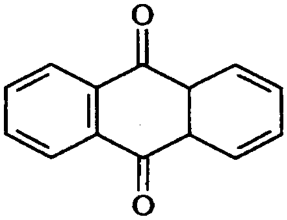
i)  ,

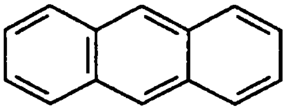
ii)  ,

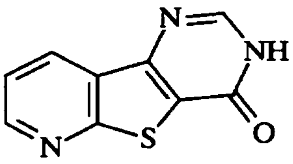
iii)  ,

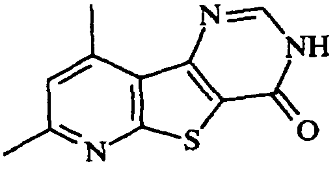
iv)  ,

v)  ,

vi)  ,

vii)  ,

viii)  , ix)



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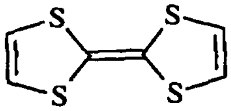

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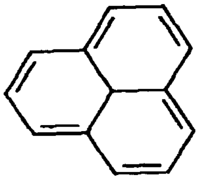

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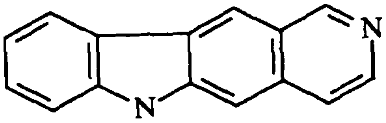

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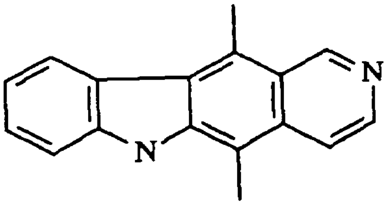

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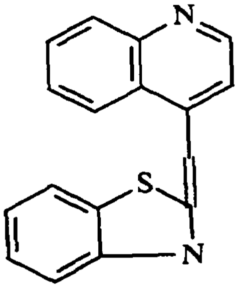

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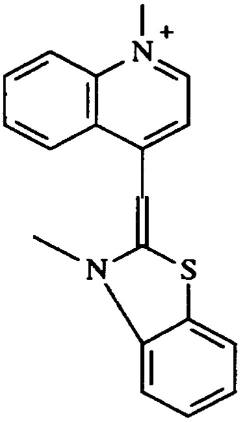

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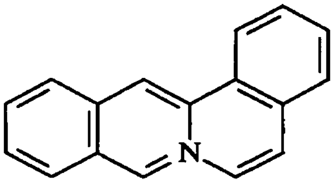

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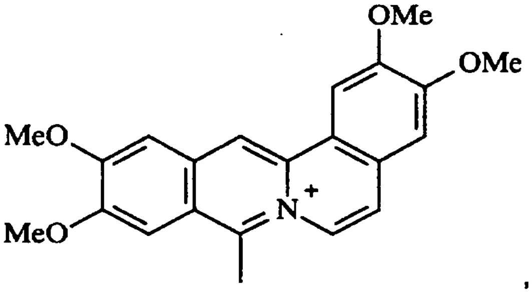

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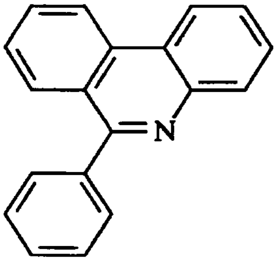

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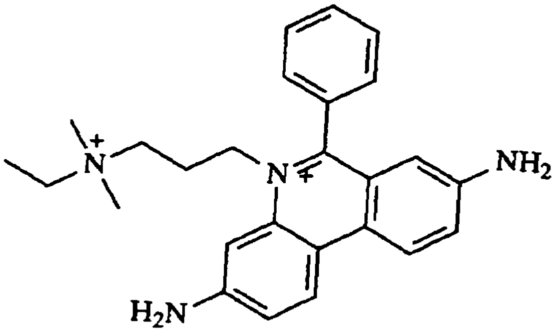

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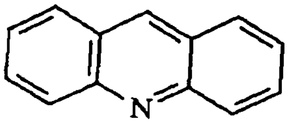

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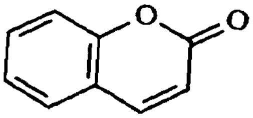

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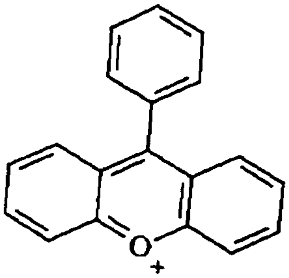

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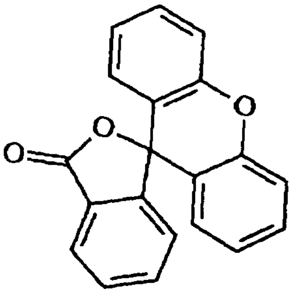

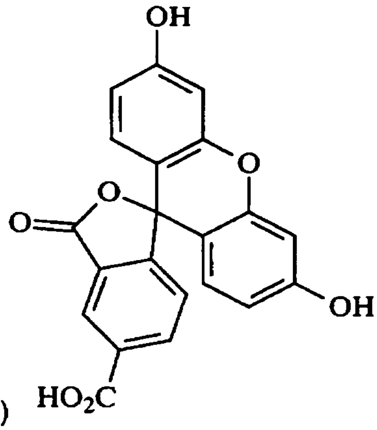

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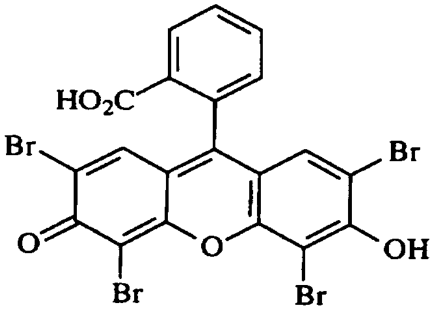

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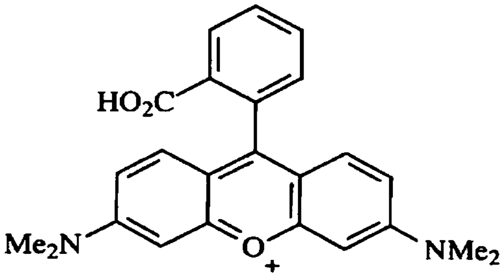

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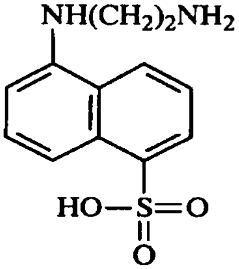

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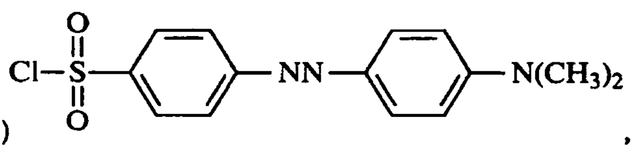

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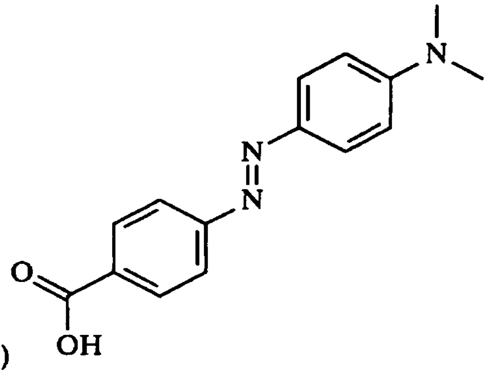

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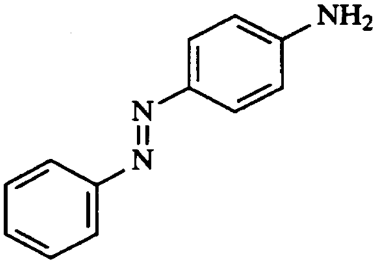

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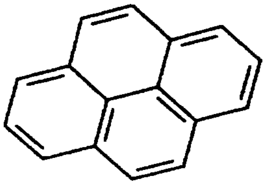
Most preferably the interacalator is pyrene of the structure



Linker

The linker of an intercalator pseudonucleotide according to the present invention is a moiety connecting the intercalator and the backbone monomer of said intercalator pseudonucleotide.

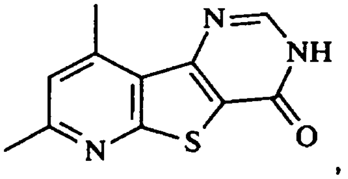
The linker usually consists of a chain of atoms or a branched chain of atoms. For example the linker may comprise a chain of x atoms selected from the group consisting of C, O, S, N. P, Se, Si, Ge, Sn and Pb, wherein one end of the chain is connected to the intercalator and the other end of the chain is connected to the backbone monomer unit.

To achieve the desired effects of the intercalator pseudonucleotides according to the present invention the total length of the linker and the intercalator should preferably be between 8 and 13 Å (see herein below). Accordingly, m should be selected dependent on the size of the intercalator of the specific intercalator pseudonucleotide.

I.e. m should be relevatively large, when the intercalator is small and x should be relatively small when the intercalator is large. For most purposes however x will be an integer from 1 to 7.

When the intercalator is pyrene, x is preferably an integer from 1 to 7, more preferably from 2 to 5, most preferably m is equal to 3.

When the intercalator has the structure:



x is preferably from 2 to 6, more preferably 2.

The chain of the linker may be substituted with one or more selected from the group consisting of C, H, O, S, N. P, Se, Si, Ge, Sn and Pb.

In one embodiment the linker is an alkyl chain. For example the linker may be an alkyl chain substituted with one or more selected from the group consisting C, H, O, S, N. P, Se, Si, Ge, Sn and Pb. In a preferred embodiment the linker consists of an unbranched alkyl chain, wherein one end of the chain is connected to the intercalator and the other end of the chain is connected to the backbone monomer unit and wherein each C is substituted with 2 H. More preferably, said unbranched alkyl chain is from 1 to 5 atoms long, such as from 1 to 3 atoms long.

In another embodiment of the invention the linker is a ring structure comprising atoms selected from the group consisting of C, O, S, N. P, Se, Si, Ge, Sn and Pb. For example the linker may be such a ring structure substituted with one or more selected from the group consisting of C, H, O, S, N. P, Se, Si, Ge, Sn and Pb.

In a preferred embodiment the linker consists of a chain of C and O atoms, wherein one end of the chain is connected to the intercalator and the other end of the chain is connected to the backbone monomer unit and wherein each C is substituted with 2 H. Preferably such a chain consists of from 1 to 6 C atoms and 1 O atom, more preferably from 1 to 3 C atoms and 1 O atom.

Examples of suitable linkers include but are not limited to the following structures:

(X)-$CH_2$-O-$CH_2$-(Q)
or
(X)-O-$CH_2$-(Q)
or
(X)-$CH_2$-$CH_2$-(Q)

Wherein X is the backbone monomer unit and Q is the intercalator and hence X and Q are not part of the linker.

## Intercalator pseudonucleotides

Intercalator pseudonucleotides according to the present invention preferably have the general structure

X-Y-Q

wherein

X is a backbone monomer unit capable of being incorporated into the backbone of a nucleic acid or nucleic acid analogue; and

Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid; and

Y is a linker moiety linking said backbone monomer unit and said intercalator.

Furthermore, in a preferred embodiment of the present invention the intercalator pseudonucleotide comprises a backbone monomer unit, wherein said backbone monomer unit is capable of being incorporated into the phosphate backbone of a nucleic acid or nucleic acid analogue in a manner so that at most 6 atoms are separating the two phosphor atoms of the backbone closest to the intercalator.

The intercalator pseudonucleotides preferably does not comprise a nucleobase capable of forming Watson-Crick hydrogen bonding. Hence intercalator pseudonucleotides according to the invention are preferably not capable of Watson-Crick base pairing.

In a preferred embodiment of the present invention, the total length of Q and Y is in the range from 7 Å to 20 Å, with the proviso that when the intercalator is pyrene the total length of Q and Y is in the range from 9 Å to 13 Å.

Preferably, the total length of Q and Y is in the range from 7 Å to 20 Å, more preferably, from 8 Å to 15 Å, even more preferably, from 8 Å to 13 Å, most preferably from 8.5 Å to 10 Å.

When the intercalator is pyrene the total length of Q and Y is preferably in the range of 8 Å to 13 Å, more preferably from 9.0 Å to 11 Å, even more preferably from 9.0 to 10 Å, most preferably around 9.8 Å.

The total length of the linker (Y) and the intercalator (Q) should be determined by determining the distance from the center of the non-hydrogen atom of the linker which is furthest away from the intercalator to the center of the non-hydrogen atom of the essentially flat, conjugated system of the intercalator that is furthest away from the backbone monomer unit. Preferably, the distance should be the maximal distance in which bonding angles and normal chemical laws are not broken or distorted in any way.

The distance should preferably be determined by a method consisting of the following steps:

a) the structure of the intercalator pseudonucleotide of interest is drawn by computer using the programme ChemWindow® 6.0 (BioRad); and

b) the structure is transferred to the computer programme SymApps™ 6.0 (BioRad); and

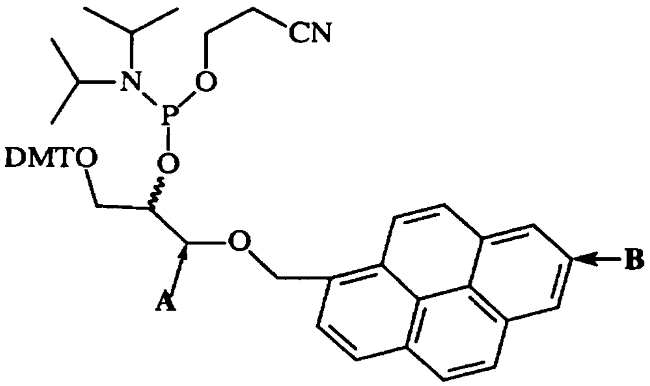
c) the 3-dimensional structure comprising calculated lengths of bonds and bonding angles of the intercalator pseudonucleotide monomer are calculated using the computer programme SymApps™ 6.0 (BioRad); and

d) the 3 dimensional structure is transferred to the computer programme RasWin Molecular Graphics Ver. 2.6-ucb; and

e) the bonds are rotated using RasWin Molecular Graphics Ver. 2.6-ucb to obtain the maximal distance (the distance as defined herein above); and

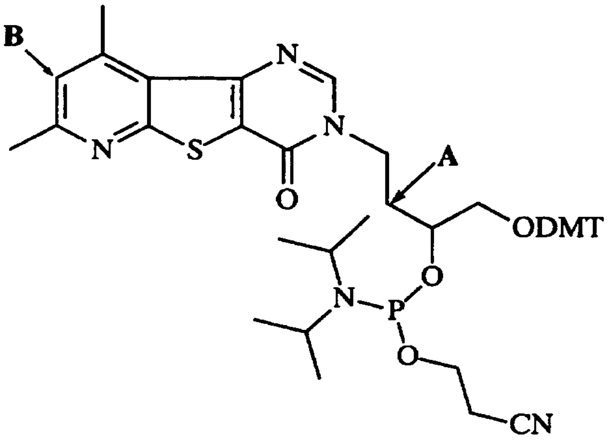
f) the distance is determined.

For example when the intercalator pseudonucleotide has the following structure:



the total length of Q and Y is determined by measuring the linear distance from A to B, which in the above example is 9,79 Å.

In another example the intercalator pseudonucleotide has the following structure:

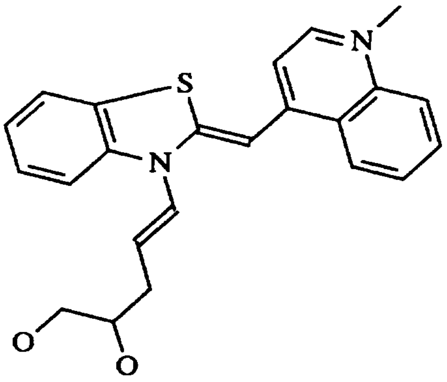


The total length of Q and Y, which is measured in a straight line from the center of the atom at A to the center of the atom at B is 8.71 Å.

Intercalator pseudonucleotides according to the present invention may be any combination of the above mentioned backbone monomer units, linkers and intercalators.

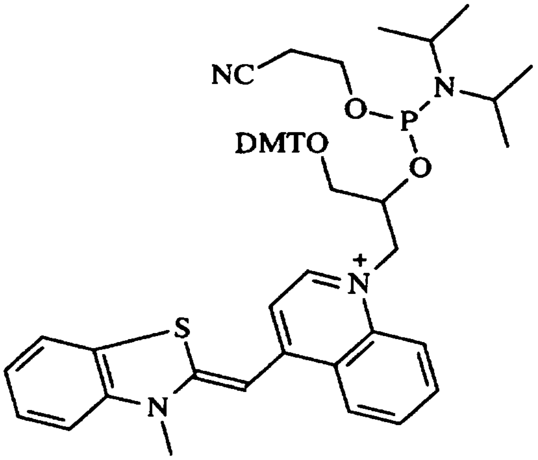
In one embodiment of the invention the intercalator pseudonucleotide is selected from the group consisting of intercalator pseudonucleotides with the structures 1) to 8 as indicated herein below:

1)



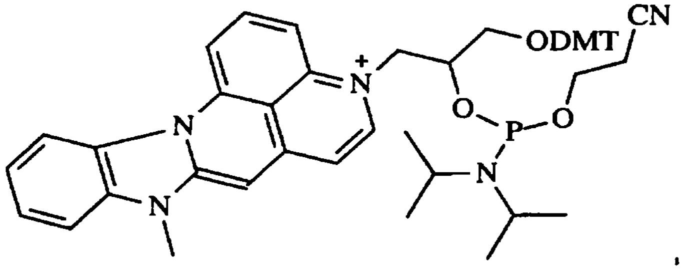
,

2)



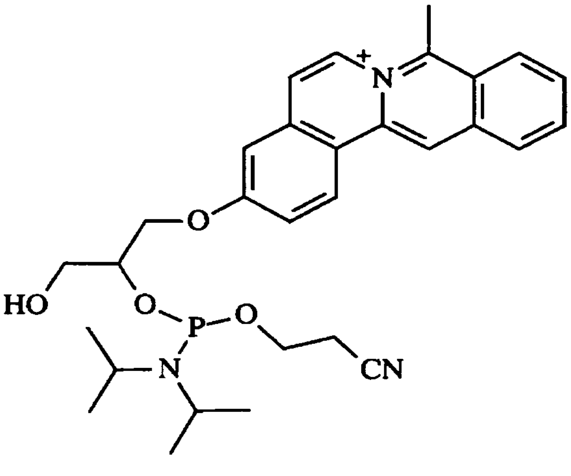
,

3)

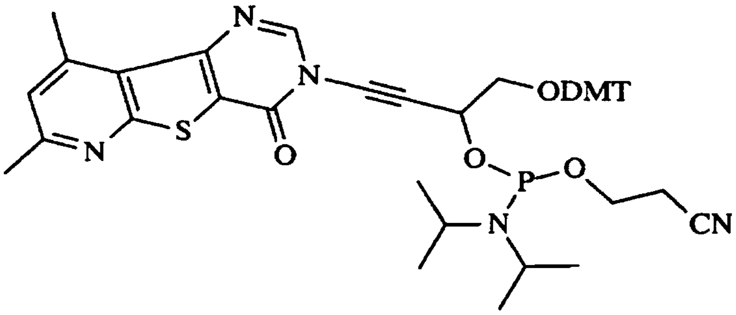


,

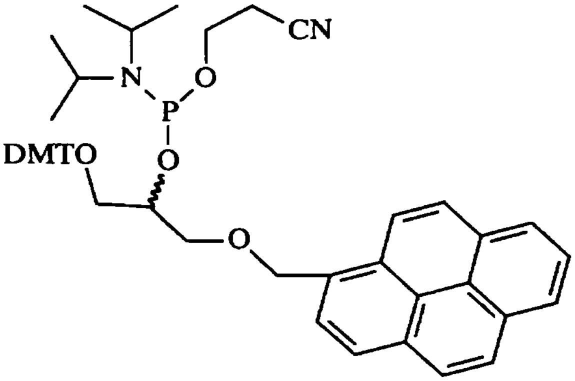
4)



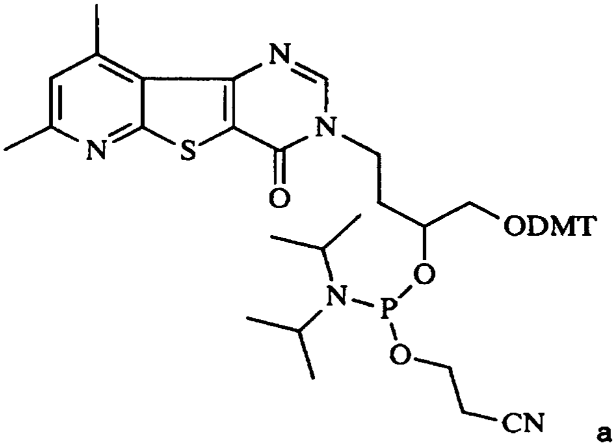
,



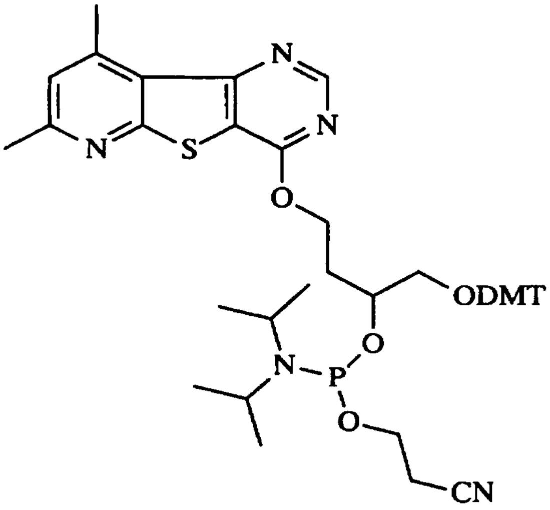
5) ,



6) ,



7) and



8)

wherein DMT and ($CH_2CH_2CN$) function as protecting groups.

In one preferred embodiment of the present invention the intercalator pseudonucleotide is the phosphoramidite of 1-(4,4'-dimethoxytriphenylmethyloxy)-3-pyrenemethyloxy-2-propanol. Even more preferably, the intercalator pseudonucleotide is selected from the group consisting of the phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethyloxy)-3-pyrenemethyloxy-2-propanol and the phosphoramidite of (R)-1-(4,4'-dimethoxytriphenylmethyloxy)-3-pyrenemethyloxy-2-propanol.

Linear oligonucleotides comprising intercalator pseudonucleotides

In one aspect the present invention relates to an oligonucleotide analogue comprising at least one intercalator pseudonucleotide of the general structure

X-Y-Q

wherein

X is a backbone monomer unit capable of being incorporated into the phosphate backbone of a nucleic acid; and

Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid; and

Y is a linker moiety linking said backbone monomer unit and said intercalator; and

wherein the oligonucleotide comprises a first sequence consisting of n nucleotides and/or nucleotide analogues and/ or intercalator pseudonucleotides and a second nucleotide sequence consisting of m nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides; and

wherein the second sequence is capable of hybridizing to a corresponding target sequence comprised within a nucleic acid or nucleic acid analogue and is capable of priming a template directed extension reaction.

The at least one intercalator pseudonucleotide may be inserted at any desired position within the first and/ or second sequence. If the oligonucleotide analogue comprises more than one intercalator pseudonucleotides, preferably every two intercalator pseudonucleotides are separated by at least one nucleotide or nucleotide analogue.

Accordingly, the intercalator pseudonucleotide may be any of the intercalator pseudonucleotides described herein above.

The first sequence preferably consists of n nucleotides and/or nucleotide analogues and intercalator pseudonucleotides, wherein n may be any suitable integer. Preferably, n is an integer in the range from 5 to 50, for example n is an integer in the range from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as from 20 to 30, for example from 30 to 50.

The second sequence preferably consists of m nucleotides and/or nucleotide analogues and/ or intercalator pseudonucleotides, wherein m may be any suitable integer. Preferably, m is an integer in the range from 5 to 50, for example n is an integer in the range from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as from 20 to 30, for example from 30 to 50.

In one preferred embodiment n is smaller than m. However it is also contained within the present invention that m is equal to n. .

The first and second sequence of the oligonucleotide analogues according to the present invention may comprise any desirable number of intercalator pseudonucleotides. For example the oligonucleotide or oligonucleotide analogue may comprise between 2 and 5, such as between 5 and 10, such as between 10 and 15, for example between 15 and 20 intercalator pseudonucleotides.

The intercalator pseudonucleotides may be placed in any desirable position within the first and the second sequence. For example, an intercalator pseudonucleotide may be placed at either end of the first and second sequence or an intercalator pseudonucleotide may be placed in an internal position within the first and second sequence. For example, the first sequence comprises at least two closely positioned intercalator pseudonucleotides.

Furthermore, if the oligonucleotide analogue comprises more than one intercalator pseudonucleotide, said pseudonucleotides may be placed in relation to each other in any desirable manner. For example, they may be placed so that 1, for example 2, such as 3, for example 4, such as 5, for example from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as more than 20 nucleotides and/ or nucleotide analogues are separating the intercalator pseudonucleotides. In a preferred embodiment, the oligonucleotide analogue comprises at least one pair of intercalator pseudonucleotides capable of forming an intramolecular excimer, intramolecular exciplex, a FRET complex and/or a charge-transfer complex.

In a preferred embodiment, the first sequence is positioned upstream of said second sequence. It is furthermore preferred that said first sequence comprises at least one pair of intercalator pseudonucleotides capable of forming an intramolecular excimer, intramolecular exciplex, a FRET complex and/or a charge-transfer complex. In a more preferred embodiment an oligonucleotide analogue comprises said first and second sequence, where said second sequence comprises only one intercalator pseudonucleotide positioned in the 5' end and where said second sequence is able to hybridize to a corresponding target sequence and prime template directed extension.

The first and second sequence of the oligonucleotide analogue may comprise more than one intercalator pseudonucleotide, wherein said intercalator pseudonucleotides may be similar or may be different.

The oligonucleotide analogues may comprise nucleotides comprised within DNA, RNA, LNA, PNA, ANA, MNA, 2'-O-methyl RNA and HNA. Accordingly, the oligonucleotide analogue may comprise one or more selected from the group consisting of subunits of DNA, RNA, LNA, PNA, ANA, MNA, 2'-O-methyl RNA and HNA, i.e. the oligonucleotide analogue may be selected from the group of DNA, RNA, LNA, PNA, ANA, MNA, 2'-O-methyl RNA, HNA and mixtures thereof.

In a preferred embodiment only the second sequence of an oligonucleotide analogue comprising a first and a second sequence according to the present invention is capable of hybridizing to a corresponding target sequence under high-stringency conditions. In a more preferred embodiment, said second sequence of said oligonucleotide analogue is only capable of hybridizing to a complementary target sequence under high-stringency conditions.

## Hairpin oligonucleotides comprising intercalator pseudonucleotides

In one aspect the present invention relates to an oligonucleotide analogue comprising at least one intercalator pseudonucleotide of the general structure

X-Y-Q

wherein

X is a backbone monomer unit capable of being incorporated into the phosphate backbone of a nucleic acid; and

Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid ; and

Y is a linker moiety linking said backbone monomer unit and said intercalator; and

wherein the oligonucleotide comprises a first sequence consisting of n nucleotides and/or nucleotide analogues and intercalator pseudonucleotides and a second nucleotide sequence consisting of m nucleotides and/or nucleotide analogues and/ or intercalator pseudonucleotides, wherein said first sequence is capable of hybridizing to said second sequence.

Accordingly, the first sequence constitutes a sequence part of the oligonucleotide analogue and the second sequence constitutes another sequence part of the oligonucleotide analogue.

The first and the second sequence may be separated by a third consisting of p nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides. Preferably, the second and third sequence are capable of hybridizing to a corresponding target sequence comprised within a nucleic acid or nucleic acid analogue and are capable of priming a template directed extension reaction.

The at least one intercalator pseudonucleotide may be inserted at any desired position within the first, second and/or third sequence. Preferably, if more than one intercalator pseudonucleotides are present, every two intercalator pseudonucleotides are separated by at least one nucleotide or nucleotide analogue.

The intercalator pseudonucleotide may be any of the intercalator pseudonucleotides described herein above.

The first sequence preferably consists of n nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides, wherein n may be any suitable integer. Preferably, n is an integer in the range from 5 to 50, for example n is an integer in the range from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as from 20 to 30, for example from 30 to 50.

The second sequence preferably consists of m nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides, wherein m may be any suitable integer. Preferably, m is an integer in the range from 5 to 50, for example n is an integer in the range from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as from 20 to 30, for example from 30 to 50.

In one preferred embodiment the first sequence may hybridize with the second sequence essentially over the entire length of the first sequence and the second sequence, even more preferably nucleotides and nucleotide analogues in the duplex are essentially complementary.

The oligonucleotide analogues according to the present invention should as a minimum be as long as the first sequence and the second sequence together, however the oligonucleotide analogues may be longer than the first sequence and the second sequence together, and accordingly the oligonucleotide analogues may comprise other parts than the first sequence and the second sequence.

For example, the oligonucleotide analogue preferably comprises between 5 and 100, such as between 5 and 10, such as between 10 and 15, for example between 15 and 20, such as between 20 and 30, for example between 30 and 40, such as between 40 and 50, for example between 50 and 60, such as between 60 and 80, for example between 60 and 100 nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides. More preferably the oligonucleotide analogue consists of in the range from 15 to 50 nucleotides.

In another embodiment, an oligonucleotide analogue according to the invention may comprise a first sequence and a second sequence, wherein said first sequence and said second sequence are separated by a third sequence consisting of p nucleotides and/or nucleotide analogues.

p may be any desirable integer, for example p may be an integer between 1 and 5, for example between 5 and 10, such as between 10 and 15, for example between 15 and 20, such as between 20 and 30, for example between 30 and 50.

The oligonucleotide analogues according to the present invention may comprise any desirable number of intercalator pseudonucleotides. For example the oligonucleotide analogue may comprise between 2 and 5, such as between 5 and 10, such as between 10 and 15, for example between 15 and 20 intercalator pseudonucleotides.

The intercalator pseudonucleotides may be positioned at any position within the first sequence of the oligonucleotide analogue.

Accordingly, the present invention relates to oligonucleotide analogues according to the present invention, wherein the first sequence comprises at least one, for example between 2 and 5, such as between 5 and 10, such as between 10 and 15, for example between 15 and 20 intercalator pseudonucleotides.

Furthermore, the invention relates to oligonucleotide analogues according to the present invention, wherein the second sequence comprises no intercalator pseudonucleotides.

The intercalator pseudonucleotides may be placed in any desirable position within the first sequence of the oligonucleotide analogues. For example, an intercalator pseudonucleotide may be placed at either end of the first sequence of the oligonucleotide analogues or an intercalator pseudonucleotide may be placed in an internal position within the oligonucleotide analogues.

Furthermore, if the oligonucleotide analogue comprises more than one intercalator pseudonucleotides, they may be placed in relation to each other in any desirable manner. For example, they may be placed so that 1, for example 2, such as 3, for example 4, such as 5, for example from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as more than 20 nucleotides are separating the intercalator pseudonucleotides. In a preferred embodiment at least two intercalator pseudonucleotides are placed so the intercalators of said intercalator pseudonucleotides are capable of forming an intramolecular excimer, an intramolecular exciplex, a intramolecular FRET complex and/or a intramolecular charge-transfer complex.

The oligonucleotide analogue may comprise more than one intercalator pseudonucleotide, wherein said intercalator pseudonucleotides may be similar or may be different.

The oligonucleotide analogues may comprise any nucleotides or nucleotide analogues. Nucleotides and nucleotide analogues can for example be chosen from the group consisting of DNA, RNA, LNA, PNA, ANA, MNA, 2'-*O*-methyl RNA and HNA. Accordingly, the oligonucleotide analogue may comprise one or more selected from the group consisting of subunits of DNA, RNA, LNA, PNA, ANA, MNA, 2'-*O*-methyl RNA and HNA, i.e. the oligonucleotide analogue may be selected from the group of DNA, RNA, LNA, PNA, ANA, MNA, 2'-*O*-methyl RNA, HNA and mixtures thereof.

In one preferred embodiment an oligonucleotide analogue comprises a first and a second sequence, where said first sequence is positioned upstream of said second sequence and where said second sequence is able to hybridize to a corresponding target sequence and prime template directed extension.

In another preferred embodiment an oligonucleotide analogue comprises a first, a second and a third sequence, where said first sequence is positioned upstream of said second and third sequence and where said second and third sequence are able to hybridize to a corresponding target sequence and prime template directed extension.

In one embodiment of the present invention it is preferred that the melting temperature of a hybrid of the first sequence and the second sequence is significantly lower than the melting temperature of a hybrid between the second sequence and a corresponding target nucleic acid or nucleic acid analogue.

In one preferred embodiment the melting temperature of a hybrid of the first and second sequence is significantly higher than the melting temperature of a hybrid between the first sequence and a non-corresponding nucleic acid or nucleic acid analogue.

In another preferred embodiment the melting temperature of a hybrid of the first and second sequence is significantly higher than the melting temperature of a hybrid between the second sequence and a non-corresponding nucleic acid or nucleic acid analogue.

In yet another preferred embodiment of the present invention the melting temperature of a hybrid of the first sequence and the second sequence is significantly lower than the melting temperature of a hybrid between the second and third sequence and a corresponding nucleic acid or nucleic acid analogue. Furthermore, the melting temperature of a hybrid of the first sequence and the second sequence may be significantly lower than the melting temperature of a hybrid between the second and a corresponding nucleic acid or nucleic acid analogue.

In a more preferred embodiment of the present invention the melting temperature of a hybrid of the first and second sequence is significantly higher than the melting temperature of a hybrid between the second and third sequence and a non-complementary nucleic acid or nucleic acid analogue.

Preferably the corresponding target nucleic acid or nucleic acid analogue is DNA or a nucleic acid analogue derived from a DNA template by extending oligonucleotides according to the present invention.

When the melting temperature of a hybrid of the first sequence and the second sequence is significantly lower than the melting temperature of a hybrid between the second and optionally third sequence and a corresponding nucleic acid or nucleic acid analogue, for example DNA, that results in the advantageous effect that in a mixture comprising the oligonucleotide analogue comprising the first sequence and the second sequence and said nucleic acid or nucleic acid analogue corresponding to the second and optionally third sequence, the second and optionally third sequence will preferably hybridize with said corresponding nucleic acid or nucleic acid analogue, rather than with the first sequence.

Accordingly, in a mixture comprising an oligonucleotide analogue according to the present invention, if the first sequence and the second sequence are hybridized, that is indicative of that only a limiting amount or for example no corresponding target nucleic acids and/ or nucleic acid analogues_are available.

Vice versa, in a mixture comprising the oligonucleotide analogues, if the first sequence and the second sequence are not hybridized, that is indicative of that the mixture furthermore comprises corresponding nucleic acids and/ or nucleic acid analogues.

Furthermore, if a limited amount of said corresponding target nucleic acids and/ or nucleic acid analogues is available, an amount of said oligonucleotide analogues corresponding to the amount of said corresponding targets will be hybridized to said corresponding targets and the remaining amount of said oligonucleotide analogue first sequences will be hybridized to said second sequences.

One non-limiting example of an hairpin oligonucleotide analogue according to the invention is given in figure 5.

## Excimer, exciplex, FRET and charge transfer

An excimer is a dimer of compounds, which is associated in an electronic excited state, and which is dissociative in its ground state. When an isolated compound_is excited it may loose its excitation or it may associate with another compound of the same kind_(which is not excited), whereby an excimer is formed. An excimer emits fluorescence at a wavelength different from monomer fluorescence emission. When the excimer looses its excitation the association is no longer favourable and the two species will dissociate. An exciplex is an excimer like dimer, wherein the two compounds_are different.

Intramolecular excimers are formed by two moieties comprised within one molecule, for example 2 polyaromatic groups within the same molecule. Similar intramolecular exciplexes are formed by two moieties comprised within one molecule, for example by 2 different polyaromatic groups.

Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon. FRET is dependent on the inverse 6th power of the intermolecular distance, making it useful over distances comparable with the dimension of biological macromolecules. Preferably the donor and the acceptor must be in close proximity (typically between 10 to 100 Å) for FRET to occur. Furthermore, the absorption spectrum of the acceptor must overlap with the fluorescence emission spectrum of the donor. It is further preferred that the donor and the acceptor transition dipole orientations must be approximately parallel.

A charge transfer complex is a chemical complex in which there is weak coordination involving the transfer of charge between two intermolecular or intramolecular moieties, called an electron donor and an electron acceptor. These two moieties exhibit an observable charge-transfer absorption band during charge-transfer transition. An example is phenoquinone, in which the phenol and quinone molecules are not held together by formal chemical bonds but are associated by transfer of charge between the compounds' aromatic ring systems.

## Spectral properties

Spectral properties, such as fluorescence, are preferred for detection of hybridisation, for example when carrying out real-time detection of for example quantitative PCR processes. Thus, fluorescent labels compatible with association to nucleic acids and nucleic acid analogues are needed.

In one preferred embodiment of the present invention, intercalator pseudonucleotides comprise fluorescence properties that may be used for detection of hybridisation and/or extension.

In another preferred embodiment monomer fluorescence of intercalator pseudonucleotides may be used to detect hybridisation or to detect the presence of extension products or to follow the real-time formation of extension products.

In a more preferred embodiment of the present invention the oligonucleotide analogue comprises a first sequence, which comprises at least two intercalator pseudonucleotides and said intercalator pseudonucleotides are capable of forming an excimer and/ or an exciplex and/ or a charge-transfer and/ or a FRET complex. Preferably said two intercalators of an oligonucleotide analogue, is placed in a distance from one another within the oligonucleotide analogue so they can interact and hence form an intramolecular excimer, an intramolecular exciplex, a FRET complex or a charge transfer complex.

In another preferred embodiment of the present invention the intercalators of at least two intercalator pseudonucleotides within said first sequence are capable of forming an intramolecular excimer, an intramolecular exciplex, an intramolecular FRET complex and/ or an intramolecular charge transfer complex, when at least one of the k nucleotides at either side of any of said intercalator pseudonucleotides are unhybridised.

More preferably the intercalators of at least two intercalator pseudonucleotides within said first sequence are capable of forming an intramolecular excimer and/ or an intramolecular exciplex and/ or an intramolecular FRET complex and/ or an intramolecular charge transfer complex, when at least one of the basepairs comprised within the k nucleotides at either side of any of said intercalator pseudonucleotides is unhybridised and said intercalators are not capable of forming an intramolecular excimer, an intramolecular exciplex, an intramolecular FRET complex or an intramolecular charge transfer complex, when all the k nucleotides at either side of any of said intercalator pseudonucleotides are hybridized.

Above, k is an integer in the range of 1 to 3.

In another preferred embodiment of the present invention the intercalators of said first sequence are capable of forming an intermolecular excimer, an intermolecular exciplex, an intermolecular FRET complex and/ or an intermolecular charge transfer complex, when the second sequence of the oligonucleotide analogue comprising interalator pseudonucleotides is not hybridized to the first sequence.

It is only possible for two molecular moieties to form an intramolecular excimer, an intramolecular exciplex, FRET or a charge transfer complex if the two molecules are positioned in relation to one another, so that they are able to interact with one another. Hence intercalators cannot form an intramolecular excimer, an intramolecular exciplex, FRET complex or a charge transfer complex if there is a molecular moiety separating the intercalators.

Intercalators according to the present invention are capable of co-stacking with nucleobases. When oligonucleotide analogues comprising said intercalators hybridize with corresponding DNA, said intercalators will preferably co-stack with the nucleobases of the hybrid. If all k nucleobases, k being an integer in the range of 1 to 3, around each of the at least two intercalators form matched base-pairs, this will preferably result in a steric hindrance of the intercalator moieties, so that said intercalators will not be able to interact and accordingly not be able to form an intramolecular excimer, an intramolecular exciplex, FRET complex or a charge transfer complex.

Accordingly, preferred oligonucleotide analogue according to the present invention are oligonucleotide analogues, wherein the first sequence comprises at least two intercalator pseudonucleotides, wherein the intercalators of said intercalator pseudonucleotides are capable of forming an intramolecular excimer, an intramolecular exciplex or a FRET complex or a charge-transfer complex, when the first sequence is not hybridized with the second sequence or another nucleic acid.

Such intercalators employed according to the present invention for example include, but is not limited to, pyrene.

The oligonucleotide analogue according to the present invention may in one embodiment comprise one or more directly or indirectly detectable labels in addition to intercalator pseudonucleotides.

In one embodiment, the oligonucleotide analogue according to the present invention may comprise fluorescent groups in addition to intercalator pseudonucleotides. The fluorescent groups may be useful for detecting the oligonucleotide analogue.

The fluorescent group may be any group capable of fluorescing, in particular any group capable of fluorescing when it is associated with said first or second sequence of the oligonucleotide analogue according to the present invention and/or when it is incorporated into a nucleic acid or nucleic acid analogue. For example the fluorescent group may be selected from the group consisting of fluorescein, FITC, rhodamine, lissamine rhodamine, rhodamine 123, coumarin, CY-2, CY-3, CY 3.5, CY-5, CY 5.5, FAM, GFP, YFP, BFP, YO-YO, HEX, JOE, Nano Orange, Nile Red, OliGreen, Oregon Green, Pico green, Radiant Red, Ribo Green, ROX, R-phycoerythrin, SYPRO Orange, SYPRO Red, SYPRO Ruby, TAMRA, Texas Red, XRITC, Propidium iodide, Acridine Orange, ethidium bromide, SYBR Gold, SYBR Green I, and SYBR Green II..

Furthermore, it is also contained within the present invention that the oligonucleotide analogue may comprise at least one quencher molecule. A quencher molecule according to the present invention is any molecule that is capable of quenching the fluorescence of a fluorescent group in its vicinity. The quencher may function by absorbing energy from the fluorescent group and dissipating the energy as heat or radiative decay. Hence, the signal from the fluorescent group will be reduced or absent. Accordingly, if a fluorescent group and a suitable quencher molecule are placed close to each other, the fluorescence of the fluorescent group will be quenched.

Examples of quencher molecules include, but are not limited to, DABCYL and TAMRA. However, the quencher molecule must be selected according to the fluorescent group.

Preferred pairs of fluorescent group-quencher molecules according to the present invention includes, but is not limited to:

| Fluorescent group | Quencher |
|---|---|
| FAM | TAMRA |
| TET | TAMRA |
| Rhodamine | TAMRA |
| Coumarin | DABCYL |
| EDANS | DABCYL |
| Fluorescein | DABCYL |
| Lucifer Yellow | DABCYL |
| Eosin | DABCYL |
| TAMRA | DABCYL |

In particular, the oligonucleotide analogue according to the present invention may comprise both a fluorescent group and a quencher molecule. The fluorescent group may be placed as dangling end in the 5' end or in the 3' end or both ends. It is also preferred that at least one quencher molecule is attached as a dangling end, more preferably all non-intercalator pseudonucleotide quencher molecules are attached as dangling ends. The quencher molecule may be placed as a dangling end in the 5' end or in the 3' end or both ends. Preferably, one the fluorescent group or one quencher molecule are placed as a dangling end, in the 5' end of the oligonucleotide analogue. Further, if one fluorescent group is placed as a dangling end in the 5' end, preferably a quencher is positioned internally. Vice versa, if one quencher molecule is placed as a dangling end in the 5' end of the oligonucleotide analogue, one fluorescent group is positioned internally.

Accordingly, the fluorescent group and the quencher molecule are positioned in relation to each other so that the quencher molecule is capable of quenching the fluorescence or part of the fluorescence of the fluorescent group when the first sequence is hybridized to the second sequence, but is not capable of quenching the fluorescence of the fluorescent group when the first sequence is not hybridized to the second sequence.

In an even more preferred embodiment an oligonucleotide analogue comprise a first sequence and a corresponding second sequence that are separated by a third sequence (a hairpin probe), where at least one of the sequences comprise at least one intercalator pseudonucleotide and the oligonucleotide analogue has an additional fluorescent group placed as dangling end in the 5' end, and quencher molecule internally positioned. Vice versa, if one quencher molecule is placed as a dangling end in the 5' end of the oligonucleotide analogue, one fluorescent group is positioned internally.

It is preferred that the quencher molecule is capable of quenching the fluorescence or part of the fluorescence of the fluorescent group when the first sequence is hybridized to the second sequence, but is not capable of quenching the fluorescence of the fluorescent group when the first sequence is not hybridized to the second sequence. In the most preferred embodiment, this is obtained when said quencher molecule and fluorescent group come into close proximity when the first and second sequences of said oligonucleotide analogue hybridize.

Accordingly it is a preferred embodiment of the present invention to provide a hairpin probe that will self-hybridize unless subjected to a fully complementary target under hybridization conditions.

When the label is a complex of at least two intercalator pseudonucleotides capable of forming an intramolecular excimer, exciplex, FRET or charge-transfer complex (see herein below) with the proviso that every two intercalator pseudonucleotides are separated by at least one nucleotide or nucleotide analogue, quenching of signal could be obtained by hybridization of the nucleotides in a region of at least one nucleotide to either side of any intercalator pseudonucleotide in the complex to a complementary sequence.

Hence it is a preferred embodiment of the present invention to provide an oligonucleotide analogue comprising at least two intercalator pseudonucleotides where the spectral properties are changed upon hybridization to a target nucleic acid or as a consequence of amplification of a target nucleic acid. In a preferred embodiment the spectral signal is low when there is no or small amounts of a target nucleic acids, and high when there is larger amounts of target nucleic acid present. When used under an amplification reaction of target sequence, e.g. by PCR, it is preferred that the spectral signal increases in correspondence to the increase of said target nucleic acid sequence.

Accordingly it is preferred to provide an oligonucleotide analogue that comprise a first sequence and a complementary second sequence that are separated by a third sequence (a hairpin probe), where second or third sequence comprise at least one intercalator pseudonucleotide and where first sequence comprise an additional complex of intercalator pseudonucleotides according to the present invention, where the spectral signal is low when said first sequence is hybridized to second sequence and high when they are not hybridized.

It is also comprised within the present invention that the first sequence may comprise a donor for FRET and the second sequence may comprise an acceptor for a FRET or vice versa. Preferably said donor and said acceptor are positioned so that FRET may occur when the first sequence is hybridized to the second sequence and accordingly FRET fluorescence may only be detected when the first sequence is hybridized to the second sequence.

FRET donor and acceptor pairs for example includes:

| Donor | Acceptor |
|---|---|
| Fluorescein | Tetramethylrhodamine |
| IAEDANS | fluorescein |
| EDANS | DABCYL |
| Fluorescein | Fluorescein |
| BODIPY FL | BODIPY FL |
| Fluorescein | QSY 7 dye |

Furthermore, hybridisation or the formation of nucleotide or nucleotide analogue extension products constituting double stranded nucleic acids and/ or nucleic acid analogues, e.g. DNA, may also be detected using a label not associated directly with the oligonucleotide analogues and optionally oligonucleotides used for priming the extension. In one embodiment the oligonucleotide analogues of the present invention are used to prime template directed extension, where after a label specific for double stranded nucleic acids and/ or nucleic acid derivatives is used to detect extension products.

In one embodiment such a label may be selected from the group consisting of, but not limited to, Propidium iodide, Acridine Orange, ethidium bromide, SYBR Gold, SYBR Green I, and SYBR Green II.

## Additional labels

In addition to fluorescent labels, oligonucleotides, oligonucleotide analogues, nucleic acids or nucleic acid analogues may be labeled by other means. This may be a preferred option if the fluorescent properties of the oligonucleotide analogues according to the present invention or other additional fluorescent lables are not used for detection. An example is when exploiting only the abilities of said oligonucleotide analogues to reduce the formation of unwanted extension products.

The most common methods of detection, not including oligonucleotide analogues with directly associated fluorescent lables, are the use of ligands that bind to labeled antibodies, the use of radioactive lables and the use of chemiluminescent agents. The choice label depends on the sensitivity, ease of conjugation with the oligonucleotide analogue, stability requirements, and available instrumentation.

Non-radioactive probes are often labeled by indirect means. Labels include antibodies, which can serve as specific binding pair members for a labeled ligand. Generally, one or more ligand molecule(s) is/are covalently bound to the probe, in this case the oligonucleotide analogue. The ligand(s) then binds to an anti-ligand molecule, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound.

Ligands and anti-ligands may be varied widely. Where a ligand has a natural antiligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, AMPPD ([3-(2'-spiroamantane )-4-methoxy-4-(3'- phosphoryloxy)-phenyl-1,2-dioxetane]) and 2,3-dihydrophthalazinediones, e.g., luminol.

Oligonucleotide analgoues may be labeled also be labeled with $^{3}H$, $^{125}I$, $^{35}S$, $^{14}C$, $^{33}P$ or $^{32}P$ and subsequently detected by autoradiography. Preferably a normal PCR including hot dNTPs is carried out, whereby only the extension product will be radioactively labeled.

The above-mentioned methods will typically require some sort of separation of extended and non-extended oligonucleotide analogues after extension of oligonucleotide analogues have been carried out, but prior to detection. The method of separation chosen will typically depend on the labeling method used and the availability of procedures and instruments. Examples of separation methods are described herein below.

## Special features of oligonucleotide analogues comprising intercalator pseudonucleotides

The high affinity of oligonucleotide analogues according to the present invention toward nucleic acids or nucleic acid analogues and the spectral properties are not the only special features possessed by this oligonucleotide analogue. In addition, several other features added by the incorporation of intercalator pseudonucleotide moieties may be exploited. The first strategy relates to the presence of intercalator pseudonucleotides, which can decrease hybridization strength of corresponding sequences, both comprising intercalator pseudonucleotides, thereby preventing cross-hybridization. The second strategy relates to the sterical hindrance posed by the intercalator pseudonucleotide, being a non-natural nucleotide, which might also inhibit a DNA or RNA polymerase of proceeding beyond an intercalator pseudonucleotide present in the template strand, enabling precise control of extension-termination. The third strategy relates to exploiting the properties of a hairpin structure to inhibit unwanted priming.

According to the first strategy, the present invention provides methods of reducing cross-hybridization between regions of individual oligonucleotide analogues comprising intercalator pseudonucleotides.

Primer-dimers are unwanted PCR extension products arising from cross-hybridization among oligonucleotides or oligonucleotide analogues used for primers in the PCR reaction. Likewise, extension products arising from cross-hybridization between other nucleic acids or nucleic acid analogues in the PCR reaction, e.g. hybridization probes, also lead to unwanted extension products. Thus, the formation of unwanted extension products is a phenomenon general to PCR methods, and in particular, it is a problem when performing PCR with many cycles. When a PCR product is intended for later use, a template extension product essentially free of DNA contaminants is often required. Therefore, the presence of unwanted extension products may impose the need for additional and time-consuming purification steps. Another example where the presence of unspecific products, especially primer-dimers, is a major concern is in quantitative PCR when using unspecific stains for detection of double stranded nucleic acids or nucleic acid analogues.

In a preferred embodiment oligonucleotide analogues according to the present invention inhibit the formation of primer-dimers by inhibiting cross-hybridization among said oligonucleotide analogues when used as primers for template directed extension.

The use of oligonucleotide analogues according to the present invention may also reduce the formation of primer-dimers even if intercalator pseudonucleotides are not capable of reducing cross-hybridization between primers. This second strategy of reducing unwanted extension products exploits that intercalator pseudonucleotides pose a sterical hindrance to polymerases as outlined below. Preferably, the first sequence of oligonucleotide analogues comprising intercalating pseudonucleotides as described herein above, are positioned in the upstream region of the oligonucleotide analogue. Another complementary, corresponding or non-corresponding oligonucleotide analogue designed the same way may be capable of hybridizing to the first mentioned oligonucleotide analogue. Upon hybridization intercalator pseudonucleotides present in the oligonucleotide analogue, serving as template for another oligonucleotide analogue priming extension, will be positioned downstream of the extension site. Since nucleic acids are extended only in the 5' to 3' direction, intercalator pseudonucleotides of said oligonucleotide analogue template will inhibit a DNA or RNA polymerase in proceeding beyond said intercalator pseudonucleotides. Therefore, only a limited number of nucleotides or nucleotide analogues will be added.

Accordingly, in one embodiment the use of oligonucleotide analogues comprising intercalator pseudonucleotides according to the present invention reduces the formation of unwanted extension products in template directed extension of nucleic acids or nucleic acid analogues by posing sterical hindrances to polymerases were extension is unwanted.

In another preferred embodiment oligonucleotide analogues comprising intercalator pseudonucleotides according to the present invention reduce the formation of unwanted extension products arising from cross-hybridization between oligonucleotides and oligonucleotide analogues in template directed extension of nucleic acids or nucleic acid analogues.

Above, said unwanted extension products refer to products arising from extension of said oligonucleotide analogues where non-target nucleic acids or nucleic acid analogues (including oligonucleotide analogues) serve as templates.

Furthermore, reduction of the formation of unwanted extension products should be interpreted as the situation compared to extension where all conditions for extension are identical except that said oligonucleotide analogues do not comprise intercalator pseudonucleotides according to the present invention.

Another strategy to reduce the formation of unwanted extension products relates to the cases where oligonucleotide analogues according to the present invention comprise a first sequence comprising intercalator pseudonucleotides, a second and a third sequence, wherein said first sequence is capable of hybridizing to said second sequence. When the first and second sequences are corresponding, they will preferably form a hairpin structure with a duplex without overhang where the 5' and 3' ends of the oligonucleotide analogue meet. The 3' end of such a structure cannot be extended, and if said structure is stable it efficiently prevents unwanted cross-hybridization to other nucleic acids or nucleic acid analogues and therefore the formation of unwanted extension products. Preferably, the melting temperature of a hybrid of said second and third sequence and a corresponding target sequence is above the melting temperature of a hybrid between the first sequence and the second sequence. Thus, when target is present, the oligonucleotide analogue will preferably hybridize with said target and prime an extension reaction. If said target is not present, said first and second sequence will hybridize forming a hairpin thus protecting from cross-hybridization and the formation of unspecific products.

In one embodiment an oligonucleotide analogue comprising intercalator pseudonucleotides according to the present invention the duplex formed between the first and second sequence is without overhang.

It is preferred that corresponding first and second sequences form duplexes, preventing unwanted extension events when the oligonucleotide analogue is not hybridized to a target sequence in a template or when oligonucleotide analogue sequences is not present or present in limiting amounts. Intramolecular duplex formation will happen fast due to the superior kinetics of the uni-molecular hybridization event thus obtained during intramolecular duplex-formation when said template is not present. When fluorescence properties of oligonucleotide analogues are used for detection of extension reactions, intercalator pseudonucleotides capable of forming intramolecular excimers, intramolecular exciplexes, FRET complexes or charge-transfer complexes can be positioned in the first sequence of said oligonucleotide analogue. By choosing this design, the fluorescence signal from said intercalator pseudonucleotides is quenched upon the formation of a duplex comprising said first and second sequence. In this way, the fast kinetics of uni-molecular hairpin formation provides means of switching between fluorescence on-off states fast. For example fast kinetics are preferred to minimize PCR cycling time.

Typically, increasing the number of different nucleic acids and/ or nucleic acid analogues in especially PCR reactions leads to the formation of a large increase in the number of unwanted extension products. This is due to the increased number of possible combinations of unspecific hybridization events between said nucleic acids and/ or nucleic acid analogues. Therefore, the methods of effectively reducing or removing unwanted extension products as described above also allows for more optimal PCR multiplexing.

## Preparation of oligonucleotide or oligonucleotide analogues

The intercalator pseudo nucleotides according to the present invention may be synthesised by any suitable method.

However preferably the method may comprise the steps of

a) providing an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of DNA coupled to an intercalator reactive group; and
b) providing a cyclic or non cyclic alkane comprising at least three linker reactive groups, which may or may not be protected; and
c) reacting said intercalator with said precursor and thereby obtaining an intercalator-precursor monomer; and
c) providing a phosphor comprising reagent ; and
d) reacting said intercalator-precursor with said phosphor comprising reagent; and
e) obtaining a intercalator pseudo nucleotide

Preferably, the intercalator reactive group is selected so that it may react with the linker reactive group. Hence, if the linker reactive group is a nucleophil, then preferably the intercalator reactive group is an electrophile, more preferably an electrophile selected from the group consisting of halo alkyl, mesyloxy alkyl and tosyloxy alkyl. More preferably the intercalator reactive group is chloromethyl. Alternatively, the intercalator reactive group may be a nucleophile group for example a nucleophile group comprising  hydroxy, thiol, selam, amine or mixture thereof.

Preferably, the cyclic or non cyclic alkane may be a polysubstituted alkane or alkoxy comprising at least three linker reactive groups. More preferably the polysubstituted alkane may comprise three nucleophilic groups such as, but not limited to, an alkane triole, an aminoalkan diol or mercaptoalkane diol. Preferably the polysubstituted alkane contain one nucleophilic group that is more reactive than the others, alternatively two of the nucleophilic groups may be protected by a protecting group. More preferably the cyclic or non cyclic alkane is 2,2-dimethyl-4-methylhydroxy-1,3-dioxalan, even more preferably the alkane is D-$\alpha$,$\beta$-isopropylidene glycerol .

Preferably, the linker reactive groups should be able to react with the intercalator reactive groups, for example the linker reactivegroups may be a nucleophile group for example selected from the group consisting of hydroxy, thiol, selam and amine, preferably a hyhroxy group. Alternatively the linker reactive group may be an electrophile group, for example selected from the group consisting of halogen, mesylates and tosylates. In a preferred embodiment at least 2 linker reactive groups may be protected by a protecting group.

The method may furthemore comprise a step of attaching a protecting group to one or more reactive groups of the intercalator-precursor monomer. For example a DMT group may be added by providing a DMT coupled to a halogen, such as Cl, and reacting the DMT-Cl with at least one linker reactive group. Accordingly, preferably at least one linker reactive group will be available and one protected. If this step is done prior to reaction with the phosphor comprising agent, then the phosphor comprising agent may only interact with one linker reactive group.

The phoshphor comprising agent may for example be a phosphoramidit, for example $NC(CH_2)_2OP(Npr^{i}{}_2)_2$ Preferably the phosphor comprising agent may be reacted with the intercalator-precursor in the presence of $N(Et)_3$ and $CH_2Cl_2$.

One specific non-limiting example of a method of synthesising an intercalator pseudonucleotide according to the present invention is outlined in example 1 and in figure 1.

Once the appropriate sequences of oligonucleotide or oligonucleotide analogue are determined, they are preferably chemically synthesised using commercially available methods and equipment: For example, the solid phase phosphoramidite method can be used to produce short oligonucleotide or oligonucleotide analogue comprising intercalator pseudonucleotides.

For example the oligonucleotides or oligonucleotide analogues may be synthesised by any of the methods described in "Current Protocols in Nucleic acid Chemistry" Volume 1, Beaucage et al., Wiley.

Oligonucleotides analogues comprising at least one intercalator pseudonucleotide according to the present invention may be synthesised according to any suitable method.

For example synthesis may comprise the steps of

a) bringing an intercalator pseudonucleotide according to the present invention into contact with a growing chain of a support-bound nucleotide, oligonucleotide, nucleotide analogue or oligonucleotide analogue; and
b) reacting said intercalator pseudonucleotide with said support-bound nucleotide, oligonucleotide, nucleotide analogue or oligonucleotide analogue; and
c) optionally further elongating said oligonucleotide analogue by adding one or more nucleotides, nucleotide analogues or intercalator pseudonucleotides to the oligonucleotide analogue in a desired sequence; and
d) cleaving said oligonucleotide analogue from said solid support; and
e) thereby obtaining said oligonucleotide analogue comprising at least one intercalator pseudonucleotide.

In one embodiment of the present invention the synthesis may comprise the steps of

a) bringing an intercalator pseudonucleotide according to the invention comprising a reactive group, which may be protected by an acid labile protection group into contact with a growing chain of a support-bound oligonucleotide or oligonucleotide analogue; and
b) reacting said intercalator pseudonucleotide with said support-bound oligonucleotide or oligonucleotide analogue; and
c) washing away excess reactants from product on the support; and
d) oxidizing the phosphite product to phosphate product; and
e) washing away excess reactants from product on support; and
f) repeating steps a)-e) until the desired number of intercalator pseudonucleotides are inserted; and
g) cleaving oligonucleotide analogue from solid support and removing base labile protecting groups in basic media; and
h) purifying oligonucleotide analogue containing acid labile protecting group; and
i) removing acid labile protecting group with acidic media; and
j) obtaining a terminus pseudonucleotide modified oligonucleotide analogue containing at least one intercalator pseudonucleotide

In another embodiment of the present invention the synthesis may comprise the steps of

a) bringing an intercalator pseudonucleotide according to the present invention into contact with an universal support; and
b) reacting said intercalator pseudonucleotide with the universal support; followed by step c) to j) as described in the method herein above.

It is also contained within the present invention that the last acid labile protection group may be removed prior to cleavage of the support-bound oligonucleotide analogue. Subsequent purification of the oligonucleotide analogue is optional.

In yet another embodiment of the present invention the method comprises the synthesis an oligonucleotide or oligonucleotide analogue comprising at least one internally positioned intercalator pseudonucleotide, wherein synthesis may comprise the steps of

a) bringing a nucleotide or nucleotide analogue protected with an acid labile protection group into contact with a growing chain of a support-bound nucleotide, oligonucleotide, nucleotide analogue or oligonucleotide analogue; and
b) reacting the protected nucleotide analogue with the growing chain of said support-bound nucleotide, oligonucleotide, nucleotide analogue or oligonucleotide analogue; and
c) washing away excess reactants from product on support; and
d) oxidizing the phosphite product to phosphate product; and
e) washing away excess reactants from product on support; and
f) removing acid labile protecting group; and
g) washing away excess reactants from product on the support; and
h) repeating steps a)-f) to obtain the desired oligonucleotide analogue sequence; and
i) cleaving the oligonucleotide analogue from solid support and removing base labile protecting groups in basic media; and
j) purifying oligonucleotide containing acid labile protecting group; and
k) removing acid labile protecting group; and

l) obtaining an intercalator modified oligonucleotide analogue.

Alternatively the last acid labile protection group may be removed prior to cleavage of the support-bound oligonucleotide analogue. Purification of the oligonucleotide analogue is optional.

## Method of detecting hybridisation

The present invention also relates to methods of detecting hybridisation between a nucleic acid or nucleic acid analogue comprising an oligonucleotide or oligonucleotide analogue and a template comprising a nucleic acid sequence capable of hybridising to the second sequence comprising the steps of

g) providing the template; and
h) providing an oligonucleotide or oligonucleotide analogue as described herein above; and
i) incubating the template and the oligonucleotide or oligonucleotide analogue under conditions allowing for hybridisation; and
j) detecting hybridisation.

The nucleic acid or nucleic acid analogue comprising an oligonucleotide or oligonucleotide analogue may in one embodiment be equal to said oligonucleotide or oligonucleotide analogue. However, it is also possible, that the nucleic acid or nucleic acid analogue comprising an oligonucleotide or oligonucleotide analogue may be longer than the oligonucleotide or oligonucleotide analogue.

For example the nucleic acid or nucleic acid analogue may be an elongated oligonucleotide or oligonucleotide analogue, wherein nucleotides have been added to the 3' end of the oligonucleotide or oligonucleotide analogue. An elongated oligonucleotide or oligonucleotide analogue may be the product of a reaction catalysed by a DNA polymerase.

In particular, a DNA polymerase may elongate an oligonucleotide or oligonucleotide analogue, when said oligonucleotide or oligonucleotide analogue is hybridised to a longer corresponding nucleic acid in a template dependent manner. Preferably said corresponding nucleic acid may be DNA or RNA and more prerably said corresponding nucleic acid might be the template.

Accordingly, in one embodiment said nucleic acid or nucleic acid analogue may be a product of a PCR reaction comprising said oligonucleotide or oligonucleotide analogue.

Hybridisation may be determined by a number of different techniques taking advantage of the properties of the oligonucleotide or oligonucleotide analogue.

In one embodiment, detection of hybridisation comprises determining spectral properties of the oligonucleotide or oligonucleotide analogue. Determination of spectral properties is described in more detail herein above.

Alternatively, hybridisation may be determined by determining the melting temperature. This may be done when the melting temperature of a hybrid between the first sequence and the second sequence is different to the melting temperature of a hybrid consisting of the second sequence and a corresponding nucleic acid or nucleic acid analogue for example the template.

The method of detecting hybridisation may be used for a number of different purposes. For example, the method may be employed for detecting hybridisation in an assay dependent on specific hybridisation, for example Southern Blotting, Northern blotting, FISH or other kinds of in situ hybridisation.

However, in a preferred embodiment the method is employed for quantification of a polymerase chain reaction (PCR).

## Method of detecting template directed extension

The present invention also relates to methods of detecting template directed extension primed by oligonucleotide analogues.

a) providing a mixture comprising the template; and
b) providing an oligonucleotide analogue as described herein above; and
c) incubating the template and the oligonucleotide analogue under conditions allowing for hybridization; and
d) incubating the template and the oligonucleotide analogue under conditions allowing for template directed extension; and
e) optionally separating extended oligonucleotide analogues from essentially non-extended oligonucleotide analogues
f) detecting extension.

For example the template may be an extended/elongated oligonucleotide or oligonucleotide analogue, wherein nucleotides have been added to the 3' end of an oligonucleotide or oligonucleotide analogue. An elongated oligonucleotide or oligonucleotide analogue may be the product of a reaction catalysed by a DNA polymerase or a ligase.

In particular, a DNA polymerase may elongate an oligonucleotide analogue, when said oligonucleotide analogue is hybridized to a longer nucleic acid or nucleic acid analogue in a template dependent manner. Preferably said nucleic acid or nucleic acid analogue may be DNA or RNA and more preferably said nucleic acid might be the template.

In one embodiment, detection of extension comprises determining spectral properties of the oligonucleotide analogue. One advantage of such a detection type is that it can be done real-time during a reaction, so that the amount of extended oligonucleotide analogue can be determined at any specific time during a reaction. The reaction may for example be a PCR.

Dependent on the kind of spectral properties that are determined, a spectral signal above or below a predetermined limit may be indicative of extension, preferably however, a spectral signal above a predetermined limit is indicative of extension.

The spectral properties may for example be fluorescent properties. In a preferred embodiment the spectral properties are monomer fluorescence and/or excimer fluorescence and/or exciplex fluorescence and/or FRET fluorescence and/or charge-transfer absorbance.

It is an embodiment of the present invention to provide oligonucleotide analogues comprising at least 2 intercalators capable of forming an intramolecular excimer and/or intramolecular exciplex and/or a FRET complex and/or charge-transfer complex as described herein above.

In an preferred embodiment said oligonucleotide analogues comprising a first sequence comprising at least 2 intercalators capable of forming an intramolecular excimer and/or intramolecular exciplex and/or FRET and/or charge-transfer complex as described herein above are employed.

## Method for detecting extension when using linear oligonucleotide analogues

In one embodiment the oligonucleotide analogue according to the present invention preferably comprises at least one intercalator pseudonucleotide capable of fluorescing, more preferably the oligonucleotide analogue comprises only intercalator pseudonucleotide capable of fluorescing.

In one embodiment monomer fluorescence of intercalator pseudonucleotides is used for detection of extension of oligonucleotide analogues according to the present invention.

In another embodiment the monomer fluorescence of an intercalator pseudonucleotide positioned at the 5' end of the second sequence, where the second sequence is positioned downstream of the first sequence, is used to detect extension of oligonucleotide analogue.

More preferably oligonucleotide analogues comprise at least 2 intercalator pseudonucleotides capable of forming an intramolecular excimer and/or intramolecular exciplex and/or FRET and/or charge-transfer complex.

In one embodiment extension of the second downstream sequence may be determined by determining the excimer, exciplex, FRET or charge-transfer fluorescence. Here high excimer, exciplex, FRET or charge-transfer fluorescence is indicative of template directed extension and low excimer, exciplex, FRET or charge-transfer fluorescence is indicative of no template directed extension.

High fluorescence is fluorescence above a predetermined limit and low fluorescence is fluorescence below a predetermined limit.

In addition it is possible to detect hybridization by determining fluorescence of one or more fluorescent groups attached to the oligonucleotide analogue, preferentially in the first sequence.

Accordingly, when fluorescence of the fluorescent group is detectable over a predetermined limit, that is indicative of no extension directed from the template, whereas when fluorescence of the fluorescent group is lower than a predetermined limit, which is indicative of no extension.

In one embodiment, said at least 2 intercalator pseudonucleotides of the first sequence are capable of co-stacking with the end of the hybrid comprised of an extended oligonucleotide analogue and a corresponding template.

In a preferred embodiment, said at least 2 intercalator pseudonucleotides of the first sequence are capable of co-stacking with the end of the hybrid comprised of an extended oligonucleotide analogue and a complementary template.

In one embodiment, non-extended oligonucleotide analogue is differentiated from extended oligonucleotide analogue by fluorescence polarization.

In another embodiment non-extended oligonucleotide analogue is differentiated from extended oligonucleotide analogue by the use of one or more fluorescent groups that give rise to a signal specific to extended oligonucleotide analogues only.

For example fluorescent dyes that are attached to the oligonucleotide analogues and are specific for double stranded nucleic acids or nucleic acid analogues may be used. When the temperature is sufficiently high allowing only corresponding hybrids of oligonucleotide analogue comprising nucleic acid analogues to hybridize, such signal may be used for detection. Signal derived from long unspecific extension products may give problems, but that may be removed by using methods for reducing unspecific hybridization and extension events as described above.

In one embodiment concerning the oligonucleotide analogue comprising a first and a second sequence, non-extended oligonucleotide analogue is separated from extended oligonucleotide analogue prior to detection.

Separation of non-extended from extended oligonucleotide analogues is only carried out when the signal from labels associated with non-extended and extended oligonucleotide analogue cannot otherwise be differentiated. Separation may for example be carried out by gel electrophoresis, chromatography, centrifugation, precipitation or any other method known to the person skilled in the art.

## Method for detecting extension and/or hybridisation when using hairpin oligonucleotide analogues

The present section concerns oligonucleotide analogues comprising a first, a second and possibly a third sequence, wherein said first sequence is capable of hybridizing to said second sequence, and wherein the first sequence may comprise at least 2 intercalators capable of forming an intramolecular excimer and/or intramolecular exciplex and/or FRET and/or charge-transfer complex as described herein above.

Accordingly, in one embodiment hybridisation of the second seqeunce and/or extension of the second sequence of the oligonucleotide analogue may be determined by determining the excimer fluorescence, exciplex fluorescence, FRET fluorescence or charge-transfer absorbance.

In addition it is possible to detect hybridization by determining fluorescence of non-intercalator pseudonucleotides attached to the oligonucleotide analogue. In particular, it is possible when oligonucleotide analogues comprise a fluorescent group and a quencher molecule as described herein above.

For example the oligonucleotide analogue may comprise a fluorescent group and a quencher molecule positioned in relation to each other so that the quencher molecule is capable of quenching the fluorescence of the fluorescent group when the first sequence is hybridized to the second sequence, but is not capable of quenching the fluorescence of the fluorescent group when the first sequence is not hybridized to the second sequence.

Accordingly, when fluorescence of the fluorescent group is detectable over a predetermined limit, that is indicative of no hybridization (i.e. no hybridization between first and second sequence) due to extension directed from the template, whereas when fluorescence of the fluorescent group is lower than a predetermined limit, that is indicative of no extension allowing hybridization (i.e. hybridization between first and second sequence).

High fluorescence is fluorescence above a predetermined limit and low fluorescence is fluorescence below a predetermined limit.

## Method of detection hybridisation and/or extension when using melting temperature

Alternatively, the presence of extension product and/or hybridisation may be determined by determining the melting temperature. This may be done when the melting temperature of a hybrid between the first sequence and the second sequence of an oligonucleotide analogue is lower than the melting temperature of an extension product of said oligonucleotide analogue and a corresponding or complementary nucleic acid or nucleic acid analogue, for example the template.

Accordingly, low melting temperature is indicative of intramolecular hybridization between first and second sequence and essentially no extension, whereas high melting temperature is indicative of the presence of extension products.

Hence a melting temperature above a predetermined limit may be indicative of extension.

In a preferred embodiment of the present invention the oligonucleotide analogue is designed in a manner so that only part of the oligonucleotide analogue is capable of hybridizing with the preferred template.

For example, in one embodiment the template does not comprise a nucleic acid sequence capable of hybridizing with the first sequence. In another embodiment the template does not comprise a nucleic acid sequence capable of hybridizing with for example 1 to 15, such as 1 to 10, for example 1 to 7, such as 1 to 5, for example 1 to 3 nucleotides flanking the intercalator pseudonucleotide(s). In yet another embodiment the template does not comprise nucleic acid sequences that may hybridize with the part of the first sequence, which is capable of hybridizing to the second sequence.

Preferably the template comprises a nucleic acid sequence capable of hybridizing with the second sequence and the third sequence, more preferably the template may comprise a nucleic acid sequence complementary to the second sequence and the third sequence.

Furthermore, the template may comprise a nucleic acid sequence capable of hybridizing with the part of the first sequence that is not capable of hybridizing to the second sequence.

Most preferably the template does not comprise a nucleic acid sequence capable of hybridizing with the first sequence and the template comprises a nucleic acid sequence capable of hybridizing with the second sequence and the third sequence.

In said embodiment, when the oligonucleotide or oligonucleotide analogue is hybridized to the template, the first sequence is not hybridized. Accordingly, when the first sequence comprises intercalators capable of forming an intramolecular excimer and/or intramolecular exciplex and/or intramolecular FRET and/or intramolecular charge-transfer complex, then excimer fluoresence and/or exciplex fluorerence and/or FRET and/or charge-transfer absorbance may be detected, when the oligonucleotide or oligonucleotide analogue is hybridized to the template.

Because it is preferred that the second sequence preferentially hybridizes with a corresponding template, the first and the second sequence will only be hybridized, when no corresponding template is present. Accordingly, when a corresponding template is available the second sequence will preferably be hybridized with said corresponding template and hence the first sequence will not be hybridized and excimer fluoresence and/or exciplex fluorerence and/or FRET and/or charge-transfer complex fluorescence above a predetermined limit may be detected.

However, in a preferred embodiment the method is employed for quantification of a polymerase chain reaction (PCR). Because the method allows for real-time detection of hybridization by determining fluorescence properties, the method allows to closely follow a PCR reaction as it is proceeding.

## Examples

The examples herein serve to illustrate specific embodiments of the invention and should not be regarded as limiting for the invention.

### Example 1

### Preparation of an intercalator pseudonucleotide

1-Pyrenemethanol is commercially available, but it is also easily prepared from pyrene by Vilsmeier-Haack formylation followed by reduction with sodium borohydride and subsequent conversion of the alcohol with thionyl chloride affords 1-(chloromethyl)pyrene in 98% yield.

The acyclic amidite **5** (fig. 1) was prepared from (S)-(+)-2,2-dimethyl-1,3-dioxalane-4-methanol and 1-(chloromethyl)pyrene in 52% overall yield. The synthesis of **5** (fig. 1) is accomplished using KOH for the alkylation reaction, and using 80% aqueous acetic acid to give the diol **3** (fig. 1), which is protected with dimethoxytritylchloride (DMT-Cl) and finally reaction with 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite affords target compound **5** (fig. 1) in 72% yield. The yield in the latter reaction step was decreased from 72% to 14% when 2-cyanoethyl *N,N*-diisopropylchlorophosphor amidite was used as the phosphitylating reagent. The synthesis of the acyclic amidite 5 is shown schematically in figure 1.

## Example 2

**Substituting a nucleotide with a 1-*O*-(pyrenylmethyl)glycerol nucleotide.**

The ODN synthesis is carried out on a Pharmacia LKB Gene Assembler Special using Gene Assembler Special software version 1.53. The pyrene amidite is dissolved in dry acetonitrile, making a 0.1 M solution and inserted in the growing oligonucleotides chain using same conditions as for normal nucleotide couplings (2 min. coupling). The coupling efficiency of the modified nucleotides is greater than 99%. The ODNs are synthesized with DMT on and purified on a Waters Delta Prep 3000 HPLC with a Waters 600E controller and a Waters 484 detector on a Hamilton PRP-1 column. Buffer A: 950 ml. 0.1 M $NH_4^+HCO_3^-$ + 50 ml MeCN pH =9.0; buffer B: 250 ml. 0.1 M $NH_4^+HCO_3^-$ + 750 ml MeCN pH = 9.0. Gradients: 5 min. 100% A, linear gradient to 100% B in 40 min., 5 min. with 100% B, linear gradient to 100% A in 1 min. and the 100% A in 29 min (product peak ≈ 37 min.). The ODNs were DMT deprotected in 925 µl $H_2O$ + 75 µl $CH_3COOH$ and purified by HPLC again using the same column, buffer system and gradients (product peak ≈ 26 min.). To get rid of the liable salts, the ODNs were re-dissolved in 1 ml of water and concentrated *in vacuo* three times.

All oligonucleotides were confirmed by MALDI-TOF analysis made on a Voyager Elite Biospectrometry Research Station from PerSeptive Biosystems. The transition state analyses were carried out on a Perkin Elmer UV/VIS spectrometer Lambda 2 with a PTP-6 temperature programmer using PETEMP rev. 5.1 software and

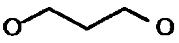
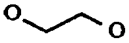
PECSS software package ver. 4.3. Melting temperature measurements of the self-complementary sequences are made in 1 M NaCl, 10 mM Na•Phosphate pH 7.0, 1.5 µM of each DNA strand. All other ODNs are measured in a 150 mM NaCl, 10 mM, Na•Phosphate, 1 mM EDTA pH 7.0, 1.5 µM of each strand. All melting temperatures giving are with an uncertainty on ± 0.5 °C.

The Amber forcefield calculations were done in MacroModel 6.0 and 7.0 with water as solvent and minimization is done by Conjugant Gradient method. The starting oligonucleotide sequences for calculation with the inserted pyrenes is taken from Brookhavens Protein Databank, and modified in MacroModel before minimazation is started. Lam and Au-Yeung solved a structure of a self-complementary sequence, equal to the one used in this work, by NMR. Their structure is prolonged with the pyrene amidite at the 5'-end of each strand and used for the structural calculations. The other sequence is a 13-mer highly conserved HIV-1 long terminal repeat region. G-7 is replaced by the pyrene amidite and calculations are made with and without an across lying C-nucleotide. The pyrene is placed in the interior of the duplex from the beginning. All bonds are free to move and to rotate.

UV melting temperature measurements (Table 2) where a G nucleotide is replaced by the flexible, abasic linkers ethylene glycol and 1,3-propandiol shows, not surprisingly, a decrease in duplex stability compared to the unmodified, fully complementary sequence. The required DMT protected cyanoethyl N,N-diisopropylphosphoramidites of ethylene glycol and 1,3-propandiol were synthesized by standard methods. Having the 1-*O*-(pyrenylmethyl)glycerol nucleotide in the same position instead of the abasic diols increases the melting temperature by 16.4 °C – 18.0 °C for the DNA/DNA duplex, indicating that the pyrene is co-axial stacking with both sides of the duplex, as the stabilization per modification exceeds the effect of placing the pyrene module at one end of a duplex (Table 1). Calculations from "MacroModel" shows that the pyrene module only makes a minor distortion of the double helix when intercalated into the duplex, having interaction with nucleobases both to the 5' side and to the 3' side of the intercalation site (Figure 3). The stabilization of the duplex by co-axial stacking of the pyrene moiety is not large enough to compensate for the loss in binding affinity due to the reduced number of hydrogen bonds by substitution of G with the pyrene moiety, the modified duplex being less stable than the unmodified fully complementary by 8.6 °C. The same trend is found for DNA/RNA duplexes although these have lower melting temperatures in general than the corresponding DNA/DNA duplexes. The stabilization of the pyrene moiety is only 8.2 °C for the DNA/RNA duplex when compared with ethylene glycol whereas the stabilization is 16.4 for the DNA/DNA duplex. The pyrene insertion results in an improved discrimination between ssDNA and ssRNA with 9.0 °C difference in the melting temperatures of their corresponding duplexes.

**Table 2** DNA/DNA and DNA/RNA duplexes where one nucleotides is either an abasic, flexible linker, the pyrene module (5), a deletion or a complementary G.

U—U/ U—U/
5' A—G—C—T—T—G—C—C—T—T—G—A—G
3' T—C—G—A—A—C—X—G—A—A—C—T—C

| Entry | | DNA $T_m$ (°C) | RNA $T_m$ (°C) | Discrimination $\Delta T_{m,\ DNA\text{-}RNA}$ (°C) |
|---|---|---|---|---|
| 1 | X =  | 26.0 | 25.8 | 0.2 |
| 2 | X =  | 27.6 | 26.8 | 0.8 |
| 3 | X = 5 | 44.0 | 35.0 | 9.0 |
| 4 | X = - (12-mer) | 35.2 | 29.6 | 4.6 |
| 5 | X = G (13-mer) | 52.6 | 47.2 | 5.4 |

## Example 3

**Fluorescence.**

A decreased fluorescence of the mono pyrene modified DNA strands upon binding to the complementary strands, indicates that the pyrene intercalates into the double helix. Double pyrene inserted oligonucleotides gives the same result for all of the different ODNs. This effect is more pronounced when the modified DNA is hybridized with ssDNA than when hybridized with ssRNA (Figures 5 and 6), indicating less intercalation of pyrene into the DNA/RNA Duplexes. This supports the conclusion from the thermal melting experiments about lacking of pyrene intercalation into the bulged DNA/RNA duplexes as deduced from the nearly identical melting temperatures with glycerol and pyrene bulges in the DNA/RNA duplexes (Table 3).

Two pyrene moieties separated by only one nucleotide generates a third peak at 480 nm, due to excimer formation of the pyrene residues. However this band is almost extinguished, when this type of DNA with two insertions with pyrene hybridizes to a complementary DNA strand. This indicates intercalation around an intact basepair preventing the two pyrene moieties to get into the physical distance of approximately 3.4 nm needed for excimer formation. When a double inserted DNA hybridizes to a complementary RNA the two pyrene moieties are still able to interact since a substantial excimer band is found.

## Example 4

### Ubeacon-primers

An example of a Ubeacon-design primer is given in figure 5. The primer consists of 39 nucleotides, which are designed so that they can form a stem-loop structure.

The primer has a target–complementary region, that is complementary to the target DNA, which is 24 nucleotides long. Furthermore, the primer has a self-complementary region, that is capable of hybridising to the other end of the primer. The self-complementary region is 15 nucleotides long and comprises furthermore 4 intercalator pseudonucleotides. Two of the intercalator pseudonucleotides are positioned so they are capable of forming an intramolecular excimer.

The melting temperature of the primer/target hybrid is around 67°C, whereas the melting temperature of the selfhybrid is around 46°C.

The Ubeacon primer can be used for PCR and allows quantification of the PCR.

Target specific Ubeacon primers and template DNA is provided (figure 6A). The Ubeacon primers and the template DNA is stored at 0-4°C.

Double stranded DNA is denatured at 94°C (figure 6B). The ubeacon primers are annealed to the target DNA at around 65°C (figure 6C) and the primers are elongated by Taq polymerase.

Subsequently, the temperature is lowered to around 45°C and unhybridised Ubeacon primers are hybridised to itself (figure 6D). Excimer fluorescence is determined and correlated to the amount of elongated Ubeacon primer.

## Example 5

## Preparation of an intercalator pseudo nucleotide

The example describes preparation and use of *N*-(pyrene-1-ylmethyl)-(3R,4R)-4-(hydroxymethyl)pyrrolidin-3-ol (**4**) in the synthesis of several INAs and investigated the hybridisation affinity of INA/DNA, INA/RNA duplexes and DNA TWJ region. When the *N*-(pyren-1-ylmethyl)azasugar was inserted as a bulge good discrimination between stabilities of INA/DNA and INA/RNA duplexes and the incresed stability of a DNA three-way junction were observed.

The synthesis of 1'-aza pyrenemethyl pseudonucleoside **4** started from enantiomerically pure 1-aza analogues of 2-deoxy-D-hexofuranose **1** or 2-deoxy-D-ribofuranose **2**.[13] Pyrene substrates having chloromethyl and carbaldehyde functionalities that could be coupled with the secondary amines **1** and **2** were used (Fig. 7).

The DMT protected phosphoroamidite **6** is required for the oligonucleotide synthesis. The primary alcohol **4** was treated with an excess of DMTCl in pyridine with further purification on a silica gel column to give compound **5** in 61% yield. The synthesis of the final phosphoroamidite by treatment with 2-cyanoethyl-*N,N*-isopropylchlorophosphoramidite in the presence of the excess of Hunig's base[12] failed. To obtain the required phosphoramidite **6** we used an alternative method with

2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphane and *N,N*-diisopropylammonium tetrazolide.[15] The compound **6** was obtained in 57% yield.

The phosphoramidite **6** was incorporated into different oligonucleotide sequences to give INAs on an automated solid phase DNA synthesizer using an increased coupling time (24 min) and repeating the cycle twice. The coupling efficiencies for the pyrene azasugar derivative **6** were approximately 80-85% compared to approximately 99% for commercial phosphoramidites (2 min coupling).

The synthesised INAs were used in the hybridisation studies of INA/DNA and INA/RNA duplexes (Fig. 8) and INA/DNA three way junction (TWJ) (Fig. 9).

INA with incorporation of the *N*-(pyren-1-ylmethyl)azasugar as the bulge resulted in lowering of the melting temperature with 1.2 °C per modification towards ssDNA (Fig. 8). The corresponding reference duplex in entry **B** containing a bulging deoxynucleotide (dG) had a considerably lower $T_m$ = 32.2 °C ($\Delta T_m$ = -10.8 °C). For the INA/RNA duplexes the pyrene containing sequence **C** and the reference **B** decreased the stability of the INA/RNA duplex with 10 °C and 9.6 °C, respectively, compared to the perfectly matched duplex (entry **A**). Consequently, INA with pyrene azasugar incorporated as the bulge has better hybridisation affinity towards the complementary ssDNA than towards ssRNA. The differences in melting temperatures for ssDNA and ssRNA seems to be additive with respect to the number of pyrene moieties in the targeting ODN. These results are also in agreement with other investigations where 1-*O*-(1-pyrenylmethyl)glycerol was inserted twice as bulges. A larger discrimination up to 25.8 °C between INA/DNA and INA/RNA was then observed.[8] In that case the INA/DNA structure is stabilised compared to the wild type duplex in contrast to our case where a slight decrease is observed for $T_m$. The flexibility of the bulge may be an important factor to obtain both duplex stabilization and discrimination. The synthesis of different linkers and planar aromatic moieties is also in progress. The RNA/DNA discrimination displayed may be applied for purification or detection of DNA targets in a mixture with the very same sequences of RNA.

DNA three way junction (TWJ) composed of two arms linked to a stem (Fig. 9), was observed to lead to a considerable stabilisation when the pyrene azasugar intercalator was inserted in the INA (**F3**) compared to the ODN having dA at the same position (**F2**) or without an insertion in the ODN (entry **F1**). To be sure that hybridization in the arms is important for the stability of the complex; we prepared ODNs with

with mismatches in either arm of TWJ (entry **E2** and **E3**). In both cases it resulted in a large lowering of the hybridisation affinity.

## Experimental

### General

NMR spectra were recorded on a Bruker AC-300 FT NMR spectrometer at 300 MHz for $^1$H NMR and at 75.5 MHz for $^{13}$C NMR. Internal standards used in $^1$H NMR spectra were TMS ($\delta$: 0.00) for $CDCl_3$, $CD_3OD$; in $^{13}$C NMR were $CDCl_3$ ($\delta$: 77.0), $CD_3OD$ ($\delta$: 49.0). Accurate ion mass determination was performed on a Kraton MS-50-RF equipped with FAB source. The $[M+H]^+$ ions were peakmatched using ions derived from the glycerol matrix. Thin layer chromatography (TLC) analyses were carried out with use of TLC plates 60 $F_{254}$ purchased from Merck and were visualized in an UV light (254 and/or 343 nm) and/or with a ninhydrin spray reagent (0.3 g ninhydrin in 100 cm$^3$ butan-1-ol and 3 cm$^3$ HOAc) for azasugars and its derivatives. The silica gel (0.063-0.200) used for column chromatography was purchased from Merck. ODNs were synthesised on an Assembler Gene Special DNA-Synthesizer (Pharmacia Biotech). Purification of 5'-*O*-DMT-on and 5'-*O*-DMT-off ODNs were accomplished using a Waters Delta Prep 4000 Preparative Chromatography System. The modified ODNs were confirmed by MALDI-TOF analysis on a Voyager Elite Elite Biospectrometry Research Station from PerSeptive Biosystems. All solvents were distilled before use. The reagents used were purchased from Aldrich, Sigma or Fluka. The reagents for Gene Assembler were purchased from Cruachem (UK).

### *N*-(Pyren-1-ylmethyl)-(3*R*,4*S*)-4-[(1*S*)-1,2-dihydroxyethyl]pyrrolidin-3-ol (3)

**Method A.** Azasugar **1** (50 mg, 0.34 mmol) was dissolved in DMF (5 cm$^3$), 1-(chloromethyl)pyrene (103 mg, 0.41 mmol) and $Et_3N$ (0.057 cm$^3$, 0.41 mmol) were added. The reaction mixture was stirred at room temp. under nitrogen overnight. The solvent was evaporated under reduced pressure and co-evaporated with toluene (2×5 cm$^3$). The residue was chromatographed on a silica gel column with $CH_2Cl_2$/MeOH (0-20%, v/v) as eluent affording the pure product **3** (70 mg, 57%): $R_f$ 0.20 (10% MeOH/$CH_2Cl_2$); $\delta_H$($CD_3OD$) 2.36 (1 H, m, H-4), 2.95 (1 H, m, H-5), 3.08 (1 H, dd, *J* 2.8 and 10.5, H-2), 3.22 (1 H, dd, *J* 5.4 and 12.0, H-5), 3.34 (1 H, m, H-2), 3.50-3.65 (3 H, m, C*H*[OH]C*H*$_2$OH), 4.42 (1 H, m, H-3), 4.65 (2 H, s, C*H*$_2$pyren-1-yl), 4.88 (3 H, br. s, 3×OH), 7.90-8.40 (9 H, m, $H_{arom}$); $\delta_C$($CD_3OD$) 50.7 (C-4), 56.7

(C-5), 57.2 (C-2), 62.7 (*C*H$_2$pyren-1-yl), 65.7 (CH$_2$OH), 71.8 (CH[OH]), 72.7 (C-3), 123.8, 125.5, 125.8, 125.9, 126.6, 126.8, 127.3, 128.0, 128.2, 129.1, 129.4, 129.9, 131.2, 131.9, 132.5, 133.2 (pyren-1-yl); *m/z* (FAB) 362.1748 $[M+H]^+$, $C_{23}H_{24}NO_3$ requires 362.1756.

**Method B.** Azasugar **1** (70 mg, 0.48 mmol) was dissolved in DMF/EtOH (3:1, 10 cm$^3$) and 1-pyrenecarbaldehyde (270 mg, 1.18 mmol) and NaCNBH$_3$ (74 mg, 1.18 mmol) were added. The reaction mixture was stirred at room temp. under nitrogen overnight. Concentrated HCl was added until pH<2. Solvent was evaporated under reduced pressure, co-evaporated with toluene (2×5 cm$^3$). The residue was purified using silica gel column chromatography with CH$_2$Cl$_2$/MeOH (0-20%, v/v) affording the compound **3** (110 mg, 63%).

### *N*-(Pyren-1-ylmethyl)-(3*R*,4*R*)-4-(hydroxymethyl)pyrrolidin-3-ol (4)

**Method A.** Azasugar **2** (100 mg, 0.86 mmol) was dissolved in DMF (10 cm$^3$) and 1-(chloromethyl)pyrene (257 mg, 1.03 mmol) and Et$_3$N (0.140 cm$^3$, 1.03 mmol) were added. The reaction mixture was stirred at room temp. under nitrogen overnight. The solvent was evaporated under reduced pressure and co-evaporated with toluene (2×5 cm$^3$). The residue was dissolved in H$_2$O/CH$_2$Cl$_2$ (1:1, 40 cm$^3$) and the water layer was extracted with CH$_2$Cl$_2$. The combined organic fractions were dried (Na$_2$SO$_4$), evaporated *in vacuo* and chromatographed on a silica gel column with CH$_2$Cl$_2$/MeOH (0-20%, v/v) affording the title compound **4** (130 mg, 46%): $R_f$ 0.17 (10% MeOH/CH$_2$Cl$_2$); $\delta_H$(CDCl$_3$) 2.08 (1 H, m, H-4), 2.29 (1 H, m, H-5), 2.57 (1 H, dd, *J* 2.8 and 10.2, H-2), 2.85 (2 H, m, H-2 and H-5), 3.43 (2 H, s, C*H*$_2$OH), 3.47 (2 H, br. s, 2×OH), 4.08 (1 H, m, H-3), 4.19 (2 H, s, C*H*$_2$pyren-1-yl), 7.90-8.40 (9 H, m, H$_{arom}$); $\delta_C$(CDCl$_3$) 49.8 (C-4), 55.9 (C-5), 57.5 (C-2), 62.3 (*C*H$_2$pyren-1-yl), 64.2 (CH$_2$OH), 73.9 (C-3), 123.3, 124.4, 124.6, 124.8, 125.1, 125.9, 127.3, 127.6, 127.8, 129.5, 130.7, 130.9, 131.1 (pyren-1-yl); *m/z* (FAB) 332.1631 $[M+H]^+$, $C_{23}H_{24}NO_3$ requires 332.1651.

**Method B.** Azasugar **2** (1.18 g, 10.1 mmol) was dissolved in DMF/EtOH (3:1, 150 cm$^3$) and 1-pyrene-carbaldehyde (3.47 g, 15.1 mmol) and NaCNBH$_3$ (950 mg, 15.1 mmol) were added. The reaction mixture was stirred at room temp. under nitrogen overnight. Concentrated HCl was added until pH<2. The solvent was evaporated under reduced pressure and co-evaporated with toluene (2×50 cm$^3$). The residue was dissolved in H$_2$O/CH$_2$Cl$_2$ (1:1, v/v, 150 cm$^3$) and the water layer was extracted with CH$_2$Cl$_2$ (3×75 cm$^3$). The combined organic fractions were dried (Na$_2$SO$_4$), evaporated under diminished pressure. The residue was purified using silica gel column chromatography with $CH_2Cl_2$/MeOH (0-20%, v/v) affording the compound **4** as an oil which crystallised on standing (1.9 g, 57%), mp 104 - 105 °C.

**Method C.** A cooled solution of compound **3** (110 mg, 0.304 mmol) in EtOH (4 cm$^3$) was added to a solution of $NaIO_4$ (71.6 mg, 0.335 mmol) in $H_2O$ (1.5 cm$^3$) under stirring. After 30 min $NaBH_4$ (12.3 mg, 0.335 mmol) was added. After 30 min the resulting solution was acidified with 2M HCl until pH 2 under vigorous stirring. The solvent was removed *in vacuo*. The residue was dissolved in $H_2O/CH_2Cl_2$ (1:1, v/v, 20 cm$^3$) and extracted with $CH_2Cl_2$ (4×15 cm$^3$). The combined organic layers were dried ($Na_2SO_4$), evaporated under diminished pressure to dryness affording compound **4** (40 mg, 40%).

### ***N*-(Pyren-1-ylmethyl)-(3*R*,4*R*)-4-[(4,4'-dimethoxytriphenylmethoxy)methyl]pyrrolidin-3-ol (5)**

Compound **4** (139 mg, 0.42 mmol) was dissolved in anhydrous pyridine (10 cm$^3$) and DMTCl (178 mg, 0.53 mmol) was added. The mixture was stirred for 24 h under nitrogen at room temp. MeOH (1 cm$^3$) was added to quench the reaction and the solvents were evaporated under reduced pressure and co-evaporated with toluene (2×5 cm$^3$). The residue was re-dissolved in $H_2O/CH_2Cl_2$ (1:1, v/v, 20 cm$^3$), and the mixture was washed with saturated aqueous $NaHCO_3$. The organic layer was dried ($Na_2SO_4$), and concentrated under reduced pressure. Purification using silica gel column chromatography (5-40% EtOAc/cyclohexane, v/v) gave the title compound **5** as a foam (160 mg, 61%) which was used in the next step without further purification: $R_f$ 0.45 (49% EtOAc/49% cyclohexane/2% $Et_3N$, v/v/v); $\delta_H(CDCl_3)$ 2.20 (1 H, m, H-4), 2.34 (1 H, m, H-5), 2.53 (1 H, br.s, OH), 2.62 (1 H, dd, *J* 5.6 and 9.9, H-2), 2.72 (1 H, dd, *J* 2.5 and 9.8, H-2), 3.06 (3 H, m, C*H*$_2$ODMT and H-5), 3.71 (6 H, s, $OCH_3$), 4.01 (1 H, m, H-3), 4.21 (2 H, s, C*H*$_2$pyren-1-yl), 6.78 (4 H, m, DMT), 7.10-7.40 (9 H, m, DMT), 7.90-8.40 (9 H, m, $H_{arom}$); $\delta_C(CDCl_3)$ 48.8 (C-4), 55.2 ($OCH_3$), 56.1 (C-5), 58.0 (C-2), 61.9 (*C*H$_2$pyren-1-yl), 64.5 ($CH_2OH$), 74.9 (C-3), 85.9 (*C*-Ar$_3$), 113.0, 123.8-132.3 (DMT and pyren-1-yl), 144.9, 158.4 (DMT); *m/z* (FAB) 634.2740 $[M+H]^+$, $C_{44}H_{42}NO_5$ requires 634.2722.

### ***N*-(Pyren-1-ylmethyl)-(3*R*,4*R*)-3-*O*-[2-cyanoethoxy(diisopropylamino)-phosphino]-4-[(4,4'-dimethoxytriphenylmethoxy)methyl]pyrrolidine (6)**

Compound **5** (140 mg, 0.22 mmol) was dissolved under nitrogen in anhydrous $CH_2Cl_2$ (5 $cm^3$). *N,N*-Diisopropylammonium tetrazolide (61 mg, 0.42 mmol) was added followed by dropwise addition of 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphane (0.140 $cm^3$, 0.44 mmol). After 2.0 h analytical TLC showed no more starting material and the reaction was quenched with $H_2O$ (1 $cm^3$) followed by addition of $CH_2Cl_2$ (10 $cm^3$). The mixture was washed with saturated aqueous $NaHCO_3$ (2×10 $cm^3$). The organic phase was dried ($Na_2SO_4$) and the solvents were removed under reduced pressure. The residue was purified using silica gel column chromatography with cyclohexane/EtOAc (0-20%, v/v). Combined UV-active fractions were evaporated *in vacuo* affording **6** (158 mg, 57%) as foam that was co-evaporated with dry acetonitrile (3×30 $cm^3$) before using it in ODN synthesis. $R_f$ 0.85 (49% EtOAc/49% cyclohexane/2% $Et_3N$, v/v/v); $\delta_H(CDCl_3)$ 0.93 (6 H, m, $CH_3$ [$Pr^i$]), 1.04 (6 H, m, $CH_3$ [$Pr^i$]), 2.30 (2 H, m, H-4 and H-5), 2.48 (2 H, m, $CH_2CN$), 2.64 (1 H, m, H-2), 2.78 (1 H, m, H-2), 2.98 (2 H, m, $OCH_2CH_2CN$), 3.08 (1 H, m, H-5), 3.50 (4 H, m, CH [$Pr^i$] and $CH_2ODMT$), 3.65 (6 H, s, $OCH_3$), 4.01 (1 H, m, H-3), 4.20 (2 H, m, $CH_2$pyren-1-yl), 6.68 (4 H, m, DMT), 7.05-7.40 (9 H, m, DMT), 7.85-8.40 (9 H, m, $H_{arom}$); $\delta_P(CDCl_3)$ 148.2 (s), 149.0 (s) in the ratio 2:1.

**Synthesis and purification of modified and unmodified oligodeoxynucleotides**

The oligodeoxynucleotides were synthesised on a Pharmacia Gene Assembler® Special synthesizer in 0.2 µmol-scale (7.5 µmol embedded per cycle, Pharmacia primer support™) using commercially available 2-cyanoethylphosphoramidites and **6**. The synthesis followed the regular protocol for the DNA synthesizer. The coupling time for **6** was increased from 2 to 24 min and the cycle was repeated twice. The 5'-O-DMT-on ODNs were removed from the solid support and deprotected with 32% aqueous $NH_3$ (1 $cm^3$) at 55 °C for 24 h and then purified on preparative HPLC using a Hamilton PRP-1 column. The solvent systems were buffer A [950 $cm^3$ 0.1 M $NH_4HCO_3$ and 50 $cm^3$ $CH_3CN$ (pH = 9.0)] and buffer B [250 $cm^3$ 0.1 M $NH_4HCO_3$ and 750 $cm^3$ $CH_3CN$ (pH = 9.0)] which were used in the following order: 5 min A, 30 min liner gradient of 0-70% B in A, 5 min liner gradient of 70-100% B in A. Flow rate was 1 $cm^3$ $min^{-1}$. The purified 5'-O-DMT-on ODNs eluted as one peak after approximately 30 min [UV control 254 nm and 343 nm (for pyrene containing ODNs)]. The fractions were concentrated *in vacuo* followed by treatment with 10% aqueous HOAc (1 $cm^3$) for 20 min and further purification on HPLC under the same conditions to afford detritylated ODNs which eluted at 23-28 min. The purity of oligos synthesised was 99-100% according to the preparative HPLC. The resulted solutions were evaporated *in vacuo* and co-evaporated twice with water to remove volatile salts to afford ODNs, which were used in melting temperature measurements. All oligonucleotides containing pyrenylmethylazasugar derivative **6** were confirmed by MALDI-TOF analysis (entry **C**: found 4005.65, calcd. 4005.76; entry **D**: 4398.02, calcd. 4398.87; entry **F3**: found 4903.05, calcd. 4904.89).

## Melting experiments

Melting temperature measurements were performed on a Perkin-Elmer UV/VIS spectrometer fitted with a PTP-6 Peltier temperature-programming element. The absorbance at 260 nm was measured from 18 °C to 85 °C in 1 cm cells. The melting temperature was determined as the maximum of the derivative plots of the melting curve. The oligodeoxynucleotides were dissolved in a medium salt buffer (pH = 7.0, 1 mM EDTA, 10 mM $Na_2HPO_4 \times 2H_2O$, 140 mM NaCl) to a concentration of 1.0 µM for each strand.

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PVS

## Claims

1. A hairpin oligonucleotide or oligonucleotide analogue comprising at least one intercalator pseudonucleotide of the general structure

   X-Y-Q

   wherein

   X is a backbone monomer unit capable of being incorporated into the phosphate backbone of a nucleic acid; and

   Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid ; and

   Y is a linker moiety linking said backbone monomer unit and said intercalator; and

   wherein the oligonucleotide comprises a first sequence consisting of n nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides and a second nucleotide sequence consisting of m nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides, wherein said first sequence is capable of hybridising to said second sequence.

2. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the first sequence and the second sequence are separated by a third sequence consisting of p nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides.

3. The oligonucleotide or oligonucleotide analogue according to claim 2, wherein the second and third sequence are capable of hybridising to a corresponding target sequence comprised within a nucleic acid or nucleic acid analogue and

wherein the second and third sequence are capable of priming a template directed extension reaction.

4. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein n is an integer in the range from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as from 20 to 30, for example from 30 to 50.

5. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein m is an integer in the range from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as from 20 to 30, for example from 30 to 50.

6. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein m is equal to n.

7. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the oligonucleotide comprises between 2 and 5, such as between 5 and 10, such as between 10 and 15, for example between 15 and 20 intercalator pseudonucleotides.

8. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the first sequence comprises at least one, for example between 2 and 5, such as between 5 and 10, such as between 10 and 15, for example between 15 and 20 intercalator pseudonucleotides.

9. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the second sequence comprises no intercalator pseudonucleotide.

10. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the first sequence comprises at least 2 intercalator pseudonucleotides, wherein the intercalators of said intercalator pseudonucleotides are capable of forming an intramolecular excimer, an intramolecular exciplex, FRET or a charge transfer complex, when the first sequence is not hybridised with the second sequence or another nucleic acid.

11. The oligonucleotide or oligonucleotide analogue according to claim 2, wherein p is an integer between 5 and 10, such as between 10 and 15, for example between 15 and 20, such as between 20 and 30, for example between 30 and 50.

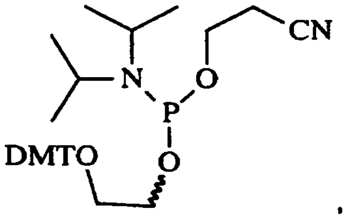
12. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the oligonucleotide consists of in the range from 15 to 50 nucleotides.

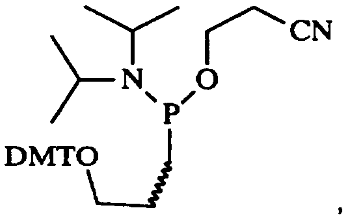
13. The oligonucelotide or oligonucleotide analogue according to claim 1, wherein at least one backbone monomer unit (X) is selected from the group consisting of acyclic backbone monomer units.

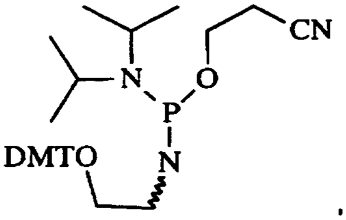
14. The oligonucelotide or oligonucleotide analogue according to claim 1, wherein at least one backbone monomer unit is selected from the group consisting of acyclic backbone monomer units capable of stabilising a bulge insertion.

15. The oligonucelotide or oligonucleotide analogue according to claim 1, wherein at least one backbone monomer unit is selected from the group consisting of phosphoramidites.

16. The oligonucelotide or oligonucleotide analogue according to claim 1, wherein the backbone monomer units are selected from the group consisting of backbone monomer units with the following structures

a)  ,

b)  , c)



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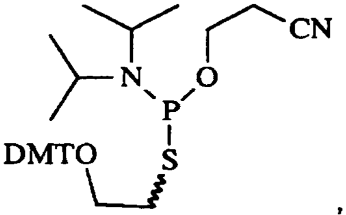
d)



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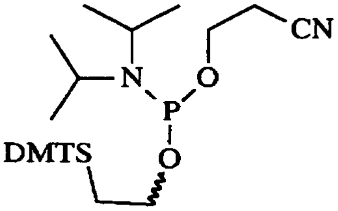
e)



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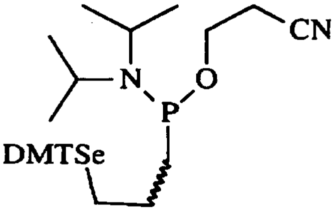
f)



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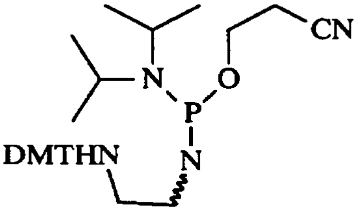

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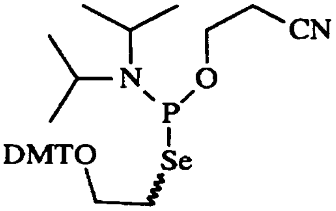

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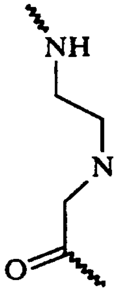
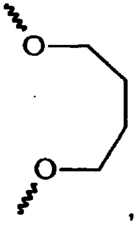
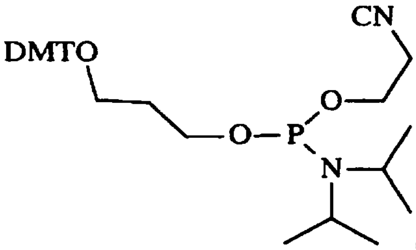
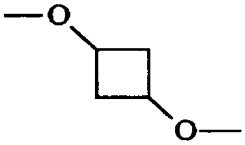
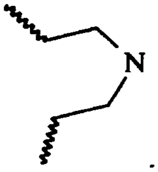
i) ,

j) ,

k) ,

l)  and

n) .

17. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the intercalators comprise a chemical group selected from the group consisting of polyaromates and heteropolyaromates.

18. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the intercalators are selected from the group consisting of polyaromates and heteropolyaromates.

19. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the intercalators are selected from the group consisting of phenanthroline, phenazine, phenanthridine, anthraquinone, pyrene, anthracene, napthene, phenanthrene, picene, chrysene, naphtacene, acridones, benzanthracenes, stilbenes, oxalo-pyridocarbazoles, azidobenzenes, porphyrins and psoralens.

20. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at least one intercalator is pyrene.

21. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the linkers comprise a chain of x atoms selected from the group consisting of C, O, S, N. P, Se, Si, Ge, Sn and Pb.

22. The oligonucleotide or oligonucleotide analogue according to claim 21, wherein x is selected from the group of integers from 1 to 7, preferably from 2 to 5, most preferably 3.

23. The oligonucleotide or oligonucleotide analogue according to claim 21, wherein the chain is substituted with one or more selected from the group consisting of C, H, O, S, N. P, Se, Si, Ge, Sn and Pb.

24. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at least one linker is an alkyl chain.

25. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at least one linker is alkyl chain substituted with one or more selected from the group consisting C, H, O, S, N. P, Se, Si, Ge, Sn and Pb.

26. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at least one linker is a ring structure comprising atoms selected from the group consisting of C, O, S, N. P, Se, Si, Ge, Sn and Pb.

27. The oligonucleotide or oligonucleotide analogue according to claim 26, wherein the linker is substituted with one or more selected from the group consisting of C, H, O, S, N. P, Se, Si, Ge, Sn and Pb.

28. The oligonucleotide or oligonucleotide analogue according 1, wherein the intercalator pseudonucleotide is the phosphor amidit of 1-(4,4'-dimethoxytriphenylmethyloxy)-3-pyrenemethyloxy-2-propanol.

29. A method of detecting hybridisation between a hairpin oligonucleotide or oligonucleotide analogue according to any of claims 1 to 28 and a template comprising a nucleic acid sequence capable of hybridising to the second sequence comprising the steps of

    a) providing the template; and
    b) providing an oligonucleotide or oligonucleotide analogue according to any of claims 1 to 28; and
    c) incubating the template and the oligonucleotide or oligonucleotide analogue under conditions allowing for hybridisation; and
    d) detecting hybridisation.

30. The method according to claim 29, wherein step d) comprises determining spectral properties of the oligonucleotide or oligonucleotide analogue.

31. The method according to claim 30, wherein a spectral signal above a predetermined limit is indicative of hybridisation.

32. The method according to claim 30, wherein the spectral properties are fluorescent properties.

33. The method according to claim 30, wherein the spectral properties are monomer fluorescence and/or excimer fluorescene and/or exciplex fluorescence and/or FRET and/or charge transfer fluorescence.

34. The method according to claim 29, wherein step d) comprises determining melting temperature.

35. The method according to claim 34, wherein a melting temperature above a predetermined limit is indicative of hybridisation.

36. The method according to claim 29, wherein the template comprises a nucleic acid sequence capable of hybridising with the second sequence and the third sequence.

37. The method according to claim 29, wherein the template does not comprises a nucleic acid sequence capable of hybridising with the first sequence.

38. A method for detecting template directed extension of an oligonucleotide analogue comprising at least one intercalator pseudonucleotides, said method comprising the steps of

    a) providing the template(s); and
    b) providing at least one oligonucleotide analogue comprising intercalator pseudonucleotide(s); and
    c) incubating said template(s) and said oligonucleotide analogue under conditions allowing for hybridization; and
    d) providing nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides; and
    e) extending the oligonucleotide analogue in a templated directed manner, thereby obtaining an extension product; and
    f) detecting said template directed extension.

39. The method according to claim 38, wherein the oligonucleotide analogue comprises a first sequence and a second sequence and wherein the second sequence is capable of hybridising with the template.

40. The method according to claim 38, wherein the oligonucleotide analogue is an oligonucleotide analogue according to any of claims 1 to 28.

41. The method according to claim 40, wherein the second sequence is capable of hybridising to the template.

42. The method according to claim 38, wherein step f) comprises determining spectral properties of the extension product.

43. The method according to claim 42, wherein a spectral signal above a predetermined limit is indicative of the presence of an extension product.

44. The method according to claim 42, wherein the spectral properties are fluorescent properties.

45. The method according to claim 42, wherein the spectral properties are monomer fluorescence and/or excimer fluorescene and/or exciplex fluorescence and/or FRET and/or charge transfer fluorescence.

46. The method according to claim 38, wherein step f) comprises determining melting temperature.

47. The method according to claim 46, wherein a melting temperature above a predetermined limit is of the presence of an extension product.

48. The method according to claim 38, wherein step e) involves the use of an enzyme selected from the group consisting of ligase and DNA polymerases.

49. The method according to claim 38, wherein the method is used in a polymerase chain reaction (PCR).

# Fig. 1

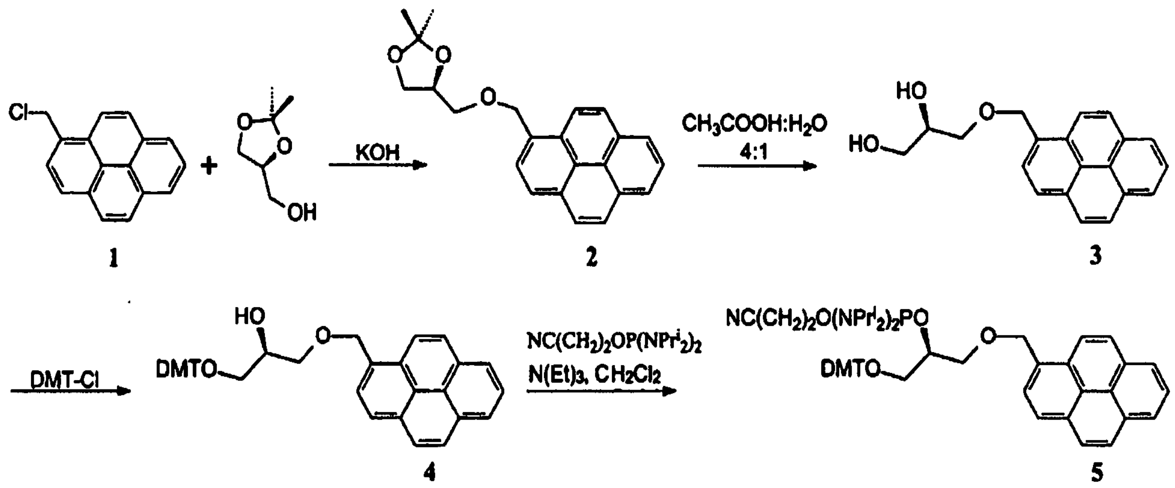

Cl
O
O
OH
KOH
O
O
O
1
2
CH3COOH:H2O
4:1
HO
HO
O
3
DMT-Cl
HO
DMTO
O
4
NC(CH2)2OP(NPri2)2
N(Et)3, CH2Cl2
NC(CH2)2O(NPri2)2PO
DMTO
O
5

Fig. 2

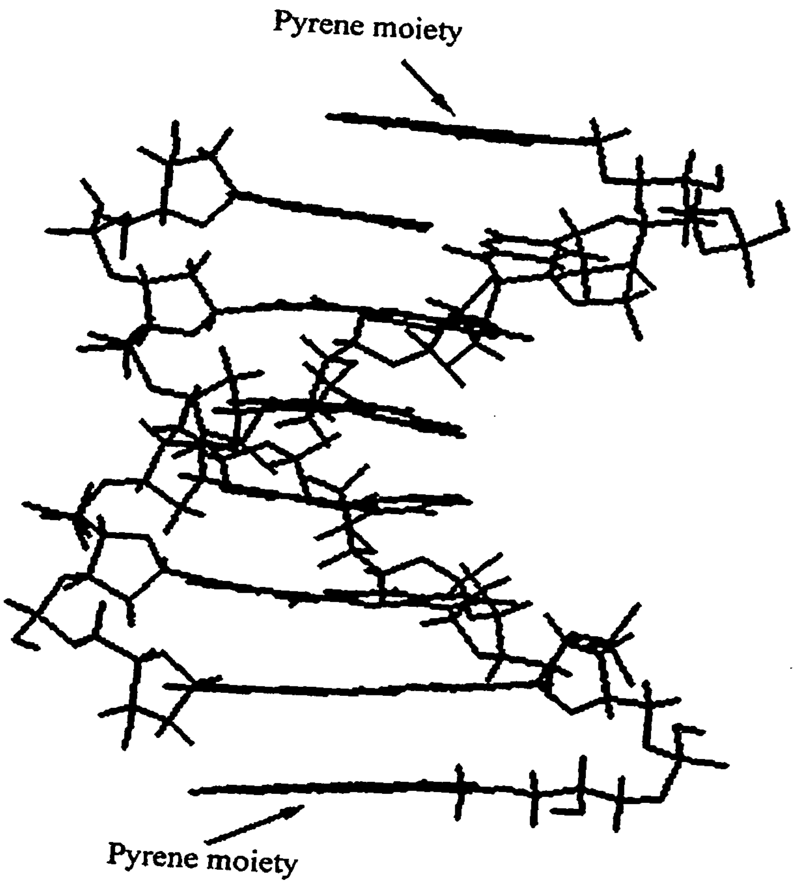


Fig. 3

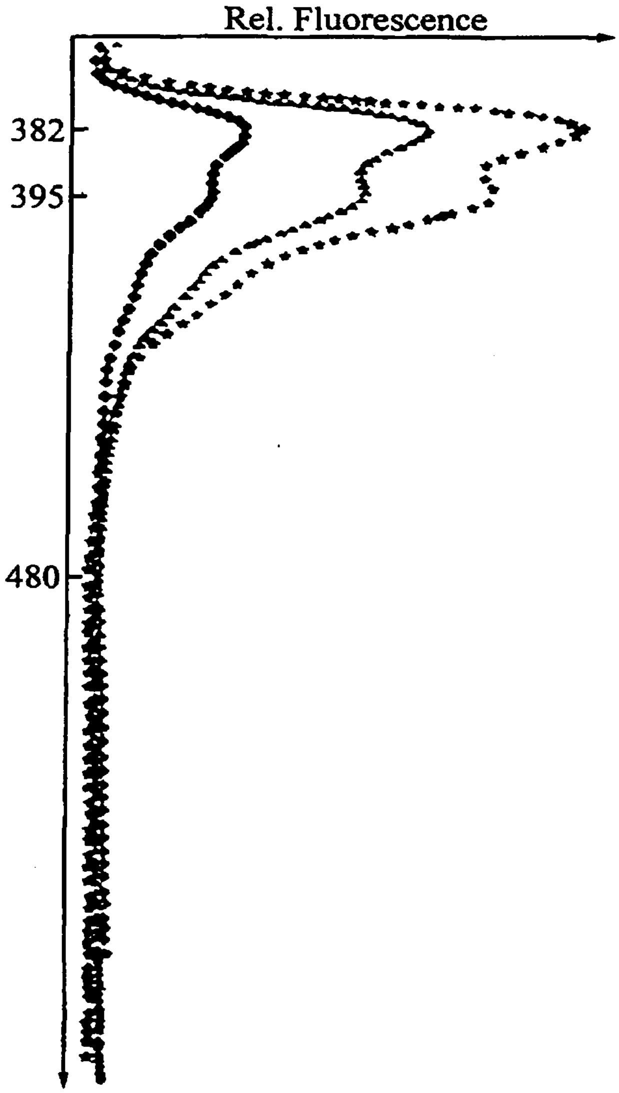
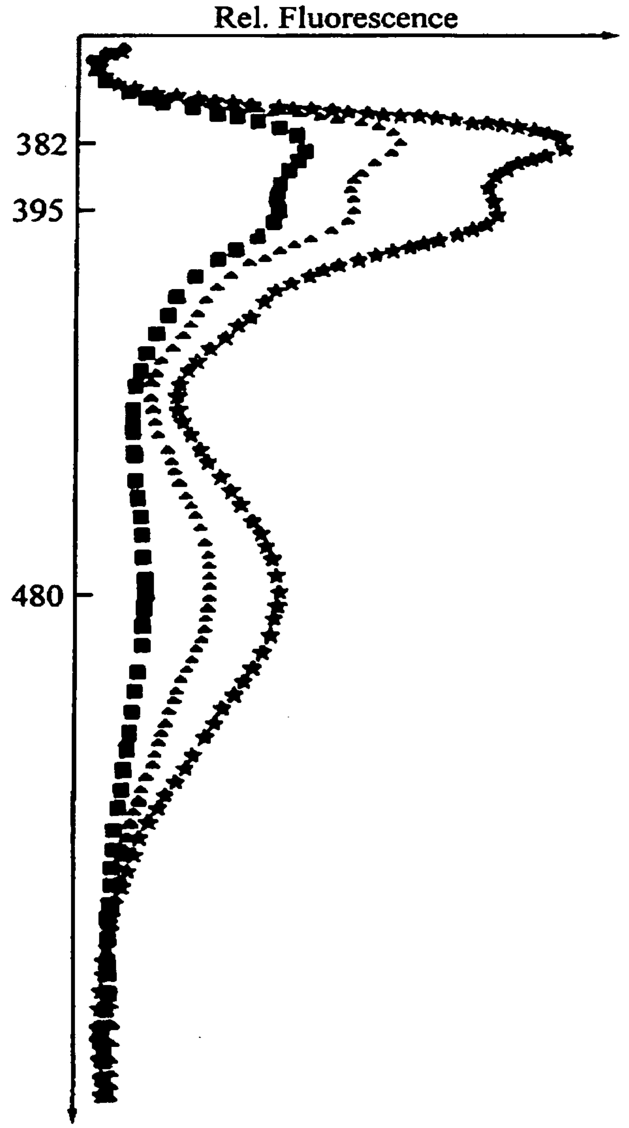
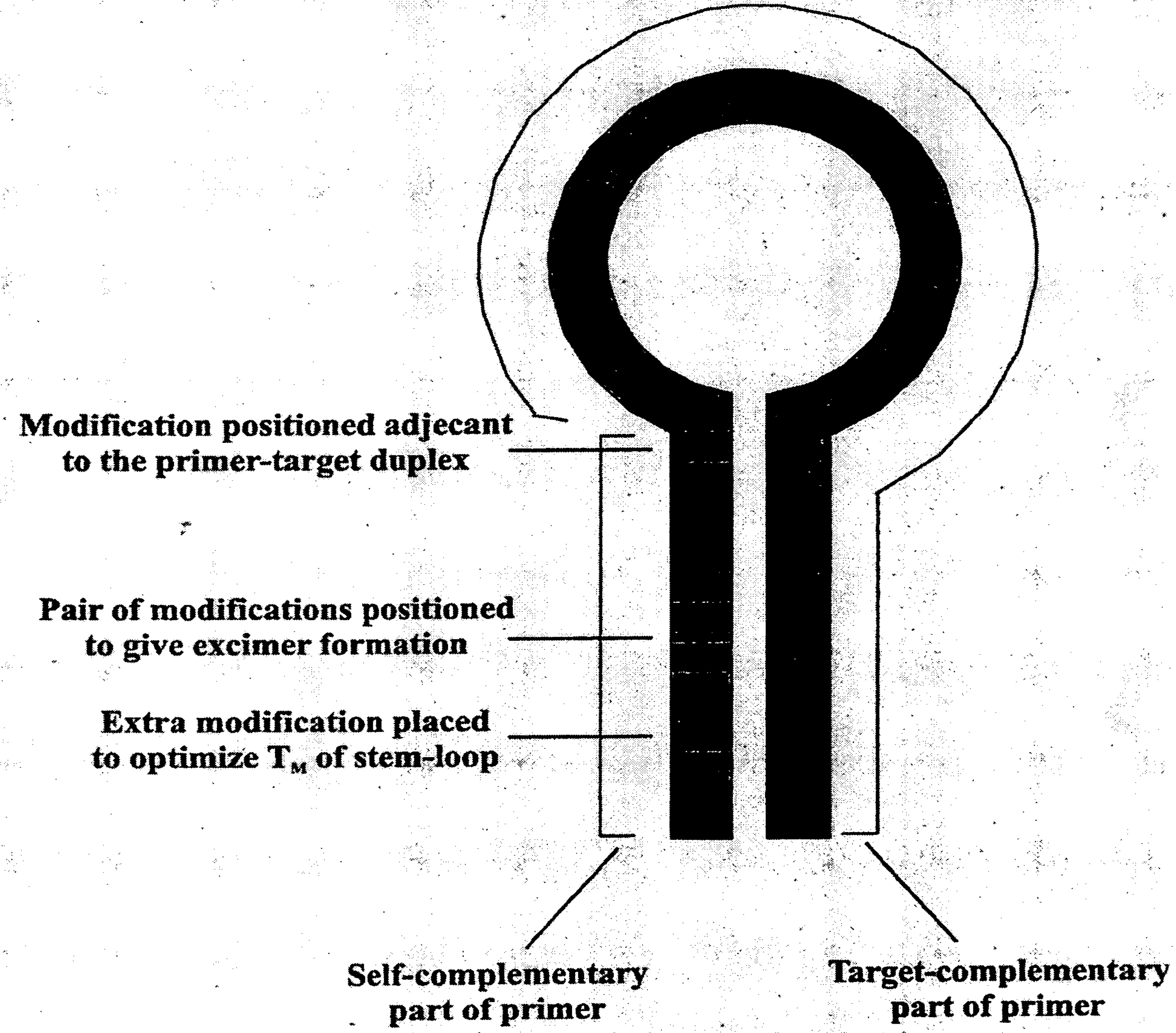


Fig. 4



Modtaget PVS
14 OKT. 2002

Fig. 5


Modification positioned adjecant to the primer-target duplex
Pair of modifications positioned to give excimer formation
Extra modification placed to optimize $T_M$ of stem-loop
Self-complementary part of primer
Target-complementary part of primer

PA 2002 01578

Fig. 6

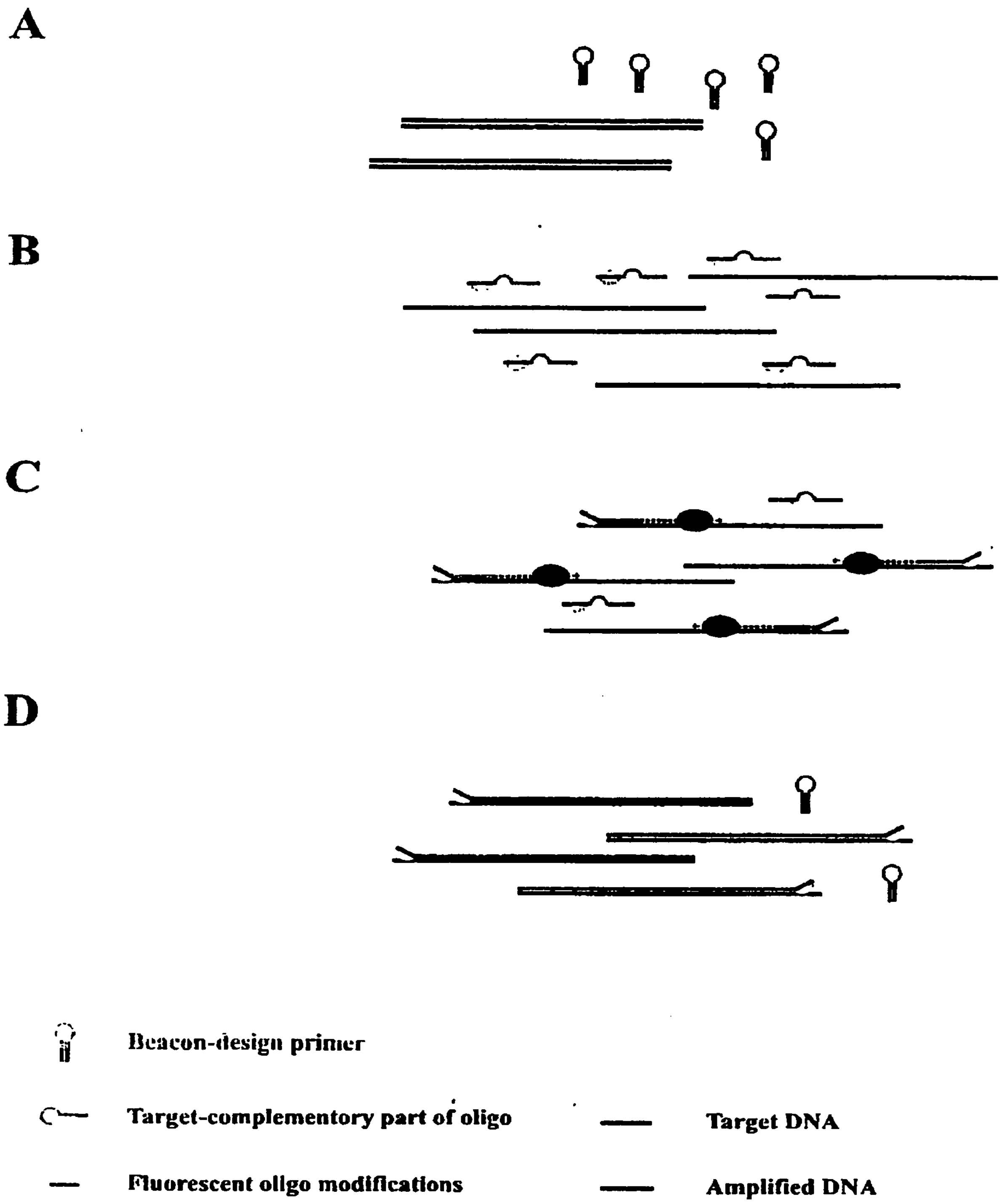
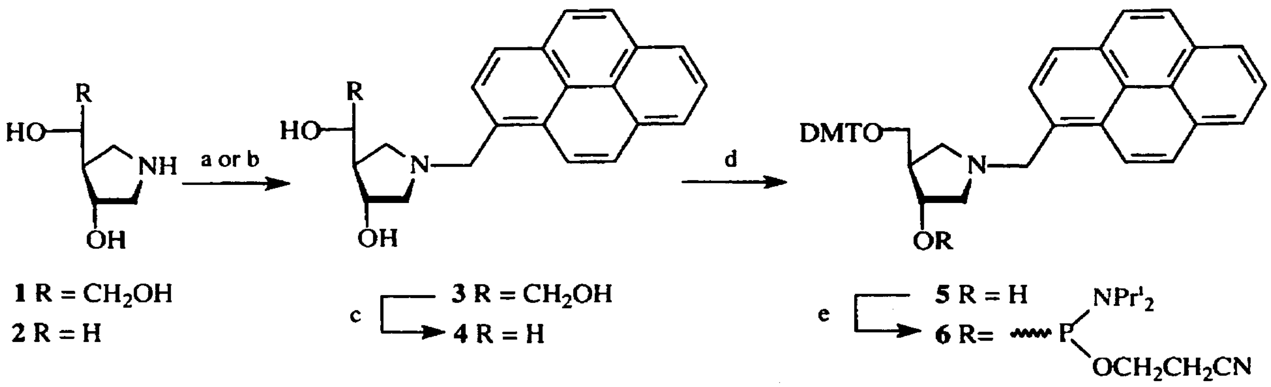

A
B
C
D
Beacon-design primer
Target-complementory part of oligo
Target DNA
Fluorescent oligo modifications
Amplified DNA

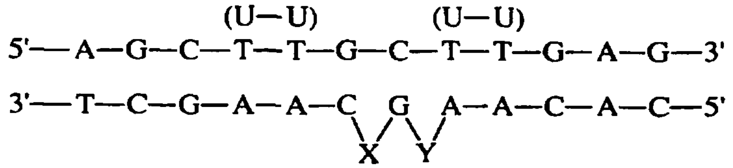
**Fig. 7**



**Scheme 1** (a) 1-(chloromethyl)pyrene, Et$_3$N, DMF; (b) 1-pyrenecarbaldehyde, NaCNBH$_3$, DMF/EtOH (3:1); (c) 1) NaIO$_4$, 2) NaBH$_4$; (d) DMTCl, pyridine; (e) NC(CH$_2$)$_2$OP(NPr$^i_2$)$_2$, *N,N*-diisopropylammonium tetrazolide,CH$_2$Cl$_2$.

Fig. 8

## Sequences and Hybridization Data of Synthesised ODNs in DNA/DNA(RNA) Duplexes

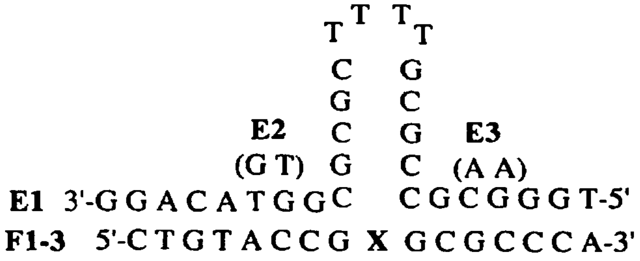


| № | X | Y | $T_m$ (°C) (DNA/DNA) | $\Delta T_m$ (°C) | $T_m$ (°C) (DNA/RNA) | $\Delta T_m$ (°C) | $\Delta\Delta T_{m\ DNA/RNA}$ |
|---|---|---|---|---|---|---|---|
| **A** | – | – | 43.0 | | 42.2 | | |
| **B** | G | – | 32.2 | -10.8 | 32.6 | -9.6 | 1.2 |
| **C** | I | – | 41.8 | -1.2 | 32.2 | -10.0 | 8.8 |
| **D** | I | I | 39.4 | -1.2 | 21.8 | -10.2 | 9.0 |

I = inserted nucleoside analogue **4**, $\Delta T_m$ = decrease in $T_m$ per modification, $\Delta\Delta T_{m\ DNA/RNA}$ = discrimination in $T_m$ between DNA/DNA and DNA/RNA duplexes per modification

**Fig. 9**

Hybridization Data ($T_m$ °C) for the DNA Three-Way Junction


T T T T
C G
G C
E2 C G E3
(G T) G C (A A)
E1 3'-G G A C A T G G C C G C G G G T-5'
F1-3 5'-C T G T A C C G X G C G C C C A-3'

| | **F1**<br>(X = 0) | **F2**<br>(X = A) | **F3**<br>(X = I) |
|---|---|---|---|
| **E1** | 38.6 | 39.4 | 48.6 |
| **E2** | <18.0 | 20.2 | 24.2 |
| **E3** | <18.0 | 19.4 | <18.0 |

I = inserted nucleoside analogue **4**